University of Alberta

IMMUNOADSORPTION FOR THE PROLONGATION OF EX VIVO CARDIAC XENOGRAFT SURVIVAL

by

Gurmeet Singh, MD

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

in

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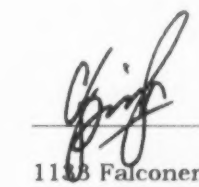

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Abstract

Immunoadsorption for the Prolongation of Ex Vivo Cardiac Xenograft Survival

Objective. Discordant xenotransplantation is complicated by hyperacute rejection (HAR), a humorally-mediated event. Human xenoreactive natural antibodies (XNA) target the αGal(1,3)βGal(1,4) moiety. Synsorb 90, an immunoadsorbent matrix bearing this carbohydrate determinant, eliminates human serum cytotoxicity to porcine endothelial cells *in vitro* by XNA depletion. Using an isolated perfused heart model, the *ex vivo* efficacy of Synsorb 90 immunoadsorption to attenuate HAR was tested.

Methods. Neonatal porcine hearts were harvested and perfused in Langendorff mode. Perfusion was terminated at 240 minutes or when the heart stopped beating. Perfusates were assessed by C3 and C4 levels, and by ELISA for XNA. Accumulation of edema was determined by the change in the mass of the heart following perfusion.

Results. Hearts perfused with pooled porcine plasma all functioned for 240 minutes (n=5), the endpoint of the study. Perfusion with pooled human plasma resulted in a 124.9 ± 96.6 (mean ± standard deviation) minute survival (n=7). Hearts perfused with human plasma treated with Chromosorb P alone, the Synsorb support matrix, had mean survival of 45.8 ± 64.5 minutes (n=5). Synsorb 90 immunoadsorption prolonged organ survival to 240 minutes in all cases (n=5), which was statistically significant ($p<0.005$). Following Synsorb 90 immunoadsorption, XNA are markedly depleted (>75% reduction, $p<0.001$). Weight change following perfusion was calculated relative to the duration of organ survival: 2.14 ± 3.53% untreated human plasma; 0.16 ± 0.04% porcine plasma; 2.95 ± 1.89% Chromosorb P; and 0.31 ± 0.05% Synsorb 90 ($p<0.005$ amongst groups).

Conclusions. These results suggest HAR is ameliorated by Synsorb 90 immunoadsorption, even in the face of complement levels sufficient to cause graft destruction. The accumulation of edema corresponds to organ failure, and thus seems to indicate the severity of rejection. The prolonged function of neonatal porcine hearts perfused with human plasma correlates with XNA depletion.

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Abbreviations and Symbols

%	Percent
°C	Degrees Celcius
α-Gal	Galα1-3Galβ1-4GlcNAc-R epitope (R=O-$(CH_2)_8$-CO-NH-BSA)
αGal(1-3)βGal(1-4)	Galα1-3Galβ1-4GlcNAc-R epitope (R=O-$(CH_2)_8$-CO-NH-BSA)
α-galactosyl	Galα1-3Galβ1-4GlcNAc-R epitope (R=O-$(CH_2)_8$-CO-NH-BSA)
µg	Microgram
µL	Microlitre
µm	Micrometre
6-MP	6-mercaptopurine
ABO	Human blood group antigen system
ADP	Adenosine diphosphate
ADPase	Adenosine diphosphatase
ANOVA	Analysis of variance
AZA	Azathioprine
BC	Before Christ
bpm	Beats per minute
BSA	Bovine serum albumin
C1	Complement component 1
C1 inh	Complement component 1 inhibitor
C1q	Complement component 1q
C2	Complement component 2
C2a	Complement component 2 fragment a
C3	Complement component 3
C3a	Complement component 3 fragment a
C3b	Complement component 3 fragment b

C4	Complement component 4
C4b	Complement component 4 fragment b
C5	Complement component 5
C5a	Complement component 5 fragment a
C5b	Complement component 5 fragment b
C5b-9	Membrane attack complex
C6	Complement component 6
C8	Complement component 8
$CaCl_2$	Calcium chloride
CD46	Membrane cofactor protein
CD55	Decay accelerating factor
CD59	Homologous restriction factor
cDNA	Complementary deoxyribonucleic acid
CHF	Congestive heart failure
cm	Centimetre
CO_2	Carbon dioxide
CP2D	Citrate-phosphate-dextrose
CsA	Cyclosporine A
CVF	Cobra venom factor
CyP	Cyclophilin
DAF	Decay accelerating factor
DNA	Deoxyribonucleic acid
DSG	15-Deoxyspergualin
DXR	Delayed xenograft rejection
EC	Endothelial cells
ECA	Endothelial cell activation
ELISA	Enzyme linked immunosorbent assay

ESRD	End-stage renal disease
Fc	Crystallizable fragment (immunoglobulin non-antigenic binding fragment)
FK506	Tacrolimus
FKBP	Tacrolimus binding protein
g	Gram
Galα(1,3)Gal	Galα1-3Galβ1-4GlcNAc-R epitope (R=O-$(CH_2)_8$-CO-NH-BSA)
gp 115/135	Triad complex of 115-kDa, 125-kDa, 135-kDa glycoproteins
gp115	115 kDa glycoprotein; β3 integrin
gp125	125 kDa glycoprotein; αIIb integrin
gp135	135 kDa glycoprotein; α2 integrin
gpIb	Platelet glycoprotein Ib
GPI 562	gpIIbIIIa antagonist
gpIIbIIIa	Platelet glycoprotein IIbIIIa
H eptiope	α-1,2-fucosyl lactosamine (O blood group antigen)
HAR	Hyperacute rejection
HCO_3^-	Bicarbonate ion
HRF	Homologous restriction factor
IκBα	NF-κB inhibitor
I.V.	Intravenous
iC3b	Complement component 3bi
ICAM-1	Intracellular adhesion molecule 1
IgA	Immunoglobulin alpha (class A)
IgG	Immunoglobulin gamma (class G)
IgG_2	Immunoglobulin gamma subclass 2
IgM	Immunoglobulin mu (class M)
IL-1	Interleukin-1

IL-2	Interleukin-2
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IMPDH	Inosine monophosphate dehydrogenase
IVC	Inferior vena cava
K^+	Potassium ion
KCl	Potassium chloride
kDa	kiloDalton
kg	Kilogram
L	Litre
LVEF	Left ventricular ejection fraction
LVSWI	Left ventricular stroke work index
M	Molar
MAbs	Monoclonal antibodies
MAC	Membrane attack complex
MCP	Membrane cofactor protein
MCP-1	Monocyte chemotactant protein-1
mEq	Milliequivalent
mg	Milligram
MHC	Major histocompatibility complex
mL	Millilitre
mmol	Millimole
MPA	Mycophenolic acid
MTT	3-4,5-dimethyldiazol-2-yl-2,5-diphenyl tetrazolium bromide
NF-κB	An ubiquitous transcription factor
NK cells	Natural killer cells

nm	Nanometre
NPC 15669	N-[9H-(2,7-dimethylfluorenyl-9-methoxy)carbonyl)-L-leucine
O.D.	Optical density
O_2	Oxygen
OKT3	Anti-CD3 monoclonal antibody
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDTC	Pyrrolidine dithiocarbamate
PGE_1	Prostaglandin E_1
pH	Logarithmic scale hydrogen ion concentration
pVCAM	Porcine vascular cell adhesion molecule
RAPA	Rapamycin
RCA	Regulators of complement activation
RNA	Ribonucleic acid
sCRI	Soluble complement receptor type 1
SDZ MTH 958	Synthetic low-molecular weight thrombin inhibitor
sialyl-Le^a	αNeuAc2-3βGAl1-3(αFuc1-4)βGlcNac-R epitope
SV	Stroke volume
SVC	Superior vena cava
Type I ECA	Type I endothelial cell activation
Type II ECA	Type II endothelial cell activation
v	volume
VADs	Ventricular assist devices
VCAM-1	Vascular cell adhesion molecule 1
vWF	von Willebrand factor
w	Weight
x g	Relative centrifugal force

XNA	Xenoreactive natural antibodies
YAC	Yeast artificial chromosome

INTRODUCTION

Human allograft transplantation has become widespread in recent years, and a concomitant shortage of donor organs has been noted. As alternatives are sought, xenotransplantation has become re-popularized in both the scientific literature, as well as the lay media. This interest in cross-species transplantation has historical roots in various cultures, far beyond this century. With an improved understanding of the immunobiology of xenograft rejection, increasing research is being conducted into the potential clinical implementation of xenotransplantation.

Alternatives to Allotransplantation

Human organ transplantation has altered the therapy and expected outcome for certain disease entities, and may be the treatment of choice for various end-stage medical conditions. However, the success of allotransplantation has been hampered by a shortage of donor organs, and 20-35% of patients listed for cardiac transplantation die while on the waiting list.[1,2] In the future, it can be expected that the demand for organs will continue to increase as the population ages. It is also likely that organ supply will diminish as innovative safety devices prevent deaths from motor vehicle accidents, whose victims have traditionally supplied many organs. The result is a resurgence of interest in identifying options to allograft transplantation.

While patients with renal insufficiency may be successfully managed by dialysis, transplantation remains the preferred treatment for end-stage renal disease (ESRD), due to the improvement in life expectancy and quality of life.[3] The specific etiology of

ESRD varies widely from diabetic nephropathy to congential disease to metabolic disorders. Ideally, then, all patients with ESRD would be treated with transplantation, except if an absolute contraindication existed.[4]

Currently, the most common indication for cardiac transplantation is cardiomyopathy of either ischemic or idiopathic etiology. Valvular disease, congential heart disease, and myocardial disorders (for example, sarcoidosis) may also be reasons to consider a heart transplant. The first successful human cardiac transplant was performed in South Africa by Barnard in 1967.[5] This historic report spurred other surgeons to follow in-suit, but the overall survival results were poor. During the 1970's, only a few centres continued with the procedure. With the development of immunosuppressive drugs, improvement in surgical technique, and the advent of endomyocardial biopsy to aid in the detection of rejection, the outcome for heart recipients improved. The criteria for acceptable donors has been broadened, in an effort to satisfy the demand for organs.[6] Research into options to cardiac transplantation has been focused in three separate areas: xenotransplantation, mechanical assist devices, and cardiomyoplasty.[7]

Studies with implantable left ventricular assist devices (VADs) have shown promise as a substitute for medical therapy - mechanical support resulted in better hemodynamics, as well as renal and liver function.[8] Although VADs may be a cost-effective intervention,[9] their use is presently limited as a bridge towards transplantation.[10] In addition, the widespread clinical application of this technology is likely to be restricted by its high cost.

The first clinical cardiomyoplasty was performed by Carpentier in 1985.[11] Cardiomyoplasty - the use of skeletal muscle power to augment ventricular function - has resulted in subjective improvements in function, but objective measures reveal small changes.[12,13] Other studies have shown improvements in functional class, left

ventricular ejection fraction, and other physiological parameters during the early postoperative period, and these hemodynamic changes were maintained at five year follow-up.[14] Multivariate analysis of these data also revealed that long-term survival was influenced by the patient's preoperative functional class and pulmonary vascular resistance.[14]

At least two multicentre prospective trials have been reported for cardiomyoplasty.[15,16] While the first study (1985-1991) reported a 21% mortality, this had decreased to 12% by the second trial (1991-1993). The survival rate, however, was the same as a prospectively-followed control group, which was matched for the degree of heart failure. Cardiac function was improved, as measured by left ventricular ejection fraction (LVEF), stroke volume (SV), and left ventricular stroke work index (LVSWI). These finding were confirmed by a quality of life assessment. Interestingly, maximal exercise capacity was not improved. In the final analysis of the second trial, one year survival following cardiomyoplasty is only 68%, which is same as natural history of congestive heart failure (CHF) without surgical intervention. The current application of this technique is solely for the treatment of CHF. The best therapy, therefore, would be heart transplantation, and xenografts may be able to fill the organ void.

Brief Historical Perspective

Man has been fascinated with the concept of interchanging organs with animals for thousands of years. It has taken until the twentieth century, however, for this to translate into a knowledge explosion in pathophysiology and immunology. There are religious scriptures that make symbolic reference to xenotransplantation.[17] For example, in the Bible, the book of Ezekiel describes four human-like creatures - the

cherubim - with wings. Their faces were those of a man, lion, ox, and eagle. The Islamic writings of the Koran relate the experiences of Lukman. Lukman's life was linked to that of an animal, and his existence can be interpreted as being symbolic of serial xenotransplantation.[17]

Various mythologies have also expressed man's desire to acquire certain animal traits. On the Indian subcontinent, Ganesha, a Hindu god, was the result of the head of an elephant transplanted onto the body of a decapitated child.[18] Reference to the origins of Ganesha date back to 1200 BC. The Greek legend of Icarus is another such story.[19] Icarus' father, Daedelus, escaped from the prison island of Crete by attaching bird feathers to his arms and flying to safety. Icarus, however, was less fortunate, as he flew too close to the sun, which melted the adhesive attaching the feathers to his arms. Perhaps this tale should serve as a warning to would-be xenografters: it may eventually be possible to successfully perform a xenograft, but the technology must be wisely applied to the clinical setting.

The use of cross-species donor grafts, therefore, is not a new concept: the first reported clinical usage of a xenograft, however, was not until 1906, when Jaboulay performed a renal transplant.[20] Since then, there have been at least thirty-five other attempted xenotransplants of kidneys and livers.[30] As well, six different investigators have undertaken cardiac xenografts on eight separate occasions.[21] Unfortunately, the immune response to a xenograft inevitably results in a vigorous response by the recipient to the new organ. All xenotransplants performed in humans have failed shortly following surgery, although one graft has survived nine months.[30]

Bailey performed a cardiac xenograft in 1984 on an infant with hypoplastic left heart syndrome, utilizing a baboon donor.[22] This was popularized in the media as "The Baby Fae Case," and the world watched with keen interest. Ultimately, the graft failed and the child died twenty days following transplantation. This case was instructive for

several reasons: firstly, hyperacute rejection was not observed. Secondly, the histopathology demonstrated little lymphocytic infiltration, suggesting that the cellular response was attenuated by the cyclosporine regimen. Furthermore, interstitial hemorrhage and coagulative necrosis was noted, implying that humoral rejection was predominant This was confirmed by antibody and complement deposition in the vasculature. Naturally, this case renewed interest in xenotransplantation.

Even more recently, Starzl's group conducted two baboon liver transplants between June 1992 and January 1993.[23] While the recipients survived for seventy and twenty-six days respectively, organ function was deemed to be suboptimal. The immunosuppressive agents employed in this trial specifically targeted both the humoral and cellular arms of the immune response. Steroids and FK506 were used to inhibit the cellular response, while prostaglandin E_1 (PGE_1) and cyclophosphamide were administered for the humoral arm. Histopathology did not reveal any humoral or cellular rejection in either case, but there was evidence for complement activation. Starzl concluded that xenotransplantation may be achievable once complement stimulation is overcome. He also suggested that conventional cellular and vascular rejection may be controlled with modern drug therapy.[24]

The next attempted liver xenograft was performed by Makowka in 1993.[25] In this case, a porcine liver was used as a bridge to support a young woman with hepatic encephalopathy until an allograft became available. Although the xenograft did function, the patient's condition continued to deteriorate, and she was declared brain dead just prior to attempting the allograft. Makowka and colleagues deduced that graft failure was the result of a rapid return of xenoreactive natural antibodes (XNA) levels following transfusion of the patient with human blood products, which was indirectly confirmed by pathological findings consistent with hyperacute rejection.[26]

Most recently, investigators at Duke University performed *ex vivo* pig-liver perfusion as a bridge towards allograft transplantation.[27] This work was modeled after an earlier xenoperfusion circuit employed by Eiseman et al.[28] Four critically-ill patients underwent venovenous perfusion through a porcine liver outside of the body. Three patients demonstrated neurological and biochemical improvement, but this was temporary, and these patients eventually succumbed to their severe hepatic dysfunction. One patient underwent four perfusion procedures and survived long enough to undergo liver transplantation with excellent result. He is presently alive and working full-time. This success has encouraged this group and others to pursue xenotransplantation.

To some extent, therefore, the rejection process in taxonomically closely-related recipient-donor combination xenotransplantation may be controllable with currently available immunosuppression - this is evident from the duration of organ survival (Appendix 1: Table 1, 2, 3). The challenge, then, remains to achieve long-term xenograft survival. In the case of distantly-related donors and recipients, this would appear to be a formidable task. There are no clinical reports in the literature of xenograft survival beyond 9 days in dissimilar donor-recipient combinations.[30] With novel approaches, and an improved understanding of the basic pathophysiological mechanisms involved in xenograft rejection, this barrier may be overcome in the near future.

Xenogeneic Donors

Xenograft donors may be characterized as either concordant or discordant.[29] Originally, these terms were used to describe the observed pattern of rejection: discordant grafts referred to combinations that result in hyperacute rejection (HAR). In the literature, however, this classification now applies to the taxonomic relationship between donor and recipient. A concordant xenograft refers to a transplant carried out between phylogenetically-close species; for example, nonhuman primates and humans. Discordant xenotransplantation denotes grafting between widely-disparate species, such as pigs and humans. Discordant donor-recipient xenografts are characterized by the presence of XNA, while concordant combinations lack preformed antibodies.

These definitions have evolved for numerous reasons.[30] Firstly, the susceptibility to HAR varies with the organ transplanted: cardiac and renal grafts are immediately revascularized and will rapidly undergo fulminant rejection. Skin and pancreatic islet grafts, however, are revascularized in a more progressive manner. Liver xenografts also appear to be more resistant to HAR, even in situations where performed circulating antibodies exist.[31] Secondly, xenografts between closely-related species may also experience HAR if major blood group incompatibility is present. When little or no XNA exist to initiate early damage, other xenogeneic antigens, especially those encoded by the major histocompatibility complex (MHC), can stimulate a later, but still potent response. That is, concordant grafts may eventually incur damage through humoral mechanisms. This was likely the mechanism of failure of concordant xenografts in previous clinical trials (Appendix: Table 1, 2, 3). In view of all this, designating species as concordant and discordant can be relevant, as it has been suggested that there is an inverse correlation between the phylogenetic distance and the likelihood of preformed XNA, with resulting HAR of grafted organs.[32]

Cost and ethical factors have led many to believe that the future of xenotransplantation will ultimately lie in discordant grafts. The practical implementation of widespread concordant xenotransplantation that relies on primate organs is severely limited by a long gestational period resulting in only a single offspring. While numerous animals have been suggested as potential donors,[33] after considering domestication, zoonoses,[34,35,36,37] and organ size-matching, porcine donors emerge as the most feasible for large-scale human usage.[38,39]

XENOGRAFT REJECTION

To date, the major obstacle to the implementation of clinical xenotransplantation remains graft rejection. The assertion has been made that the term "natural immunity" should be used to describe discordant xenograft rejection.[40] Natural immunity refers to natural antibodies and complement, while rejection is a process that may occur without the recipient having been previously exposed to donor antigens. For the purposes of this paper, however, rejection, natural immunity, and xenoimmunity will be employed interchangeably to describe the processes involved in xenograft failure.

The stages of xenograft failure have been classified as hyperacute rejection (HAR), delayed xenograft rejection (DXR), or the T-cell mediated phase. Temporally, HAR occurs within minutes to hours of graft revascularization, and represents a violent response to the foreign graft.[41] If the organ is able to elude the HAR response, DXR occurs after a few days. DXR represents a form of acute vascular rejection occurring days to weeks following engraftment. T-cell mediated graft rejection occurs even later in the life of the xenograft, although some researchers are now beginning to assert that the T-cell response may be more important than previously thought.[42,43,44,45,46] Interestingly, T-cell responses across the xenogeneic barrier may not be as potent as the T-cell response in human allograft rejection.[47]

In order to modify the HAR response, it is imperative to understand the key immunological components and mechanisms activated. Specifically, XNA bind the donor endothelium, initiating the complement cascade which inflicts damage on the organ. Therefore, the XNA and complement systems deserve examination. The concept of endothelial cell activation (ECA) is also important to both HAR and DXR, and will be discussed below.

Xenoreactive Natural Antibodies & Hyperacute Rejection

Porcine donors appear to be an ideal source of discordant xenogeneic organs.[38,39] The large number of animals currently bred and consumed would indicate that utilization of this species is ethically acceptable. Porcine organs are large enough to be useful for grafting into human recipients, while lacking the potential morbidity of zoonotic organisms that may be harbored by concordant primate donors.[34,35,36,37] Another advantage of the pig is multiple gestation, producing many conceivable donors. However, like other discordant species, porcine organs are also susceptible to HAR.[25,26]

All mammals have circulating natural antibodies against blood group antigens,[48] and these antibodies are able to elicit HAR of renal and cardiac allografts.[49,50] It was postulated that similar antibodies may initiate agglutination of xenogeneic red blood cells, thus contributing to HAR.[51,52]

The data supporting HAR initiation from XNA activity is compelling.[53] Of the primates, humans, apes, and Old World monkeys possess natural anti-porcine antibodies.[54,55] These are preformed circulating XNA that recognize oligosaccharide determinants on swine endothelial cell surfaces. It is known that the XNA in humans possess a carbohydrate specificity to the Galα1-3Galβ1-4GlcNAc-R structure (R=O-$(CH_2)_8$-CO-NH-bovine serum albumin, which is expressed on porcine endothelial cells.[56,57,58] This epitope is variously denoted in the literature as αGal(1-3)βGal(1-4), Galα(1,3)Gal, or simply α-Gal. Although this determinant is especially prominent on vascular endothelium, it is widely distributed on pig tissues.[59]

Galili proposed that some evolutionary force, likely an infectious agent containing the Galα1-3Galβ1-4GlcNAc-R epitope, caused inactivation of the α1,3-galactosyltransferase gene.[54] Neutralization of this gene obviated production of the α-galactosyl epitopes, and the human host was then free to produce antibodies against pathogens containing this epitope (anti-α-galactosyl antibodies). Coincidentally, these

xenoreactive natural antibodies also recognize carbohydrate moieties on porcine cell surfaces, triggering HAR.[60] Some reports also imply that anti-porcine XNA may demonstrate cytotoxicity to cells even in the absence of the typical vascular events that culminate in HAR.[61]

Galili's hypothesis regarding the evolution of anti-α-Gal antibodies as a response to pathogens was confirmed in a series of elegant experiments by Makowka's group.[62,63] A monoclonal antibody of IgM class was found to be capable of producing HAR in a hamster-rat model. Following characterization, this antibody was adsorbed against a panel of synthetic carbohydrates, and a number of oligosaccharides were noted to be reactive. The strongest reaction was against the sialyl-Le^a epitope, a receptor for E and P-selectins. The role of selectins in xenograft rejection is discussed below ("Endothelial Cell Activation"). Suffice it to say that xenoantibody binding to this epitope will stimulate leukocyte recruitment and adhesion. Further analysis revealed that this monoclonal antibody was coded for by a gene in a germline configuration. Similar genes code for polyreactive natural antibodies directed against infectious organisms. These investigators concluded that anti-porcine XNA were the result of sensitization of the human host to the carbohydrate structure of bacterial cell walls, such that XNA represent a primitive humoral B-cell response. XNA production, this group argued, is an early, T-cell independent response to potential pathogens.

In human beings, up to 1% of circulating antibody exhibited anti-α-Gal binding activity.[64] Thus, a relatively minute fraction of antibody results in a particularly fulminant rejection reaction. Investigators have found variability in cytotoxicity and XNA titres upon examination of a pool of human serum.[65] Due to the large variance of XNA between individuals, studies examining the role of XNA should employ a pool of human blood products. IgG and IgM binding to porcine endothelial surfaces has been shown to directly correlate with the degree of cytotoxicity, but total immunoglobulin levels were not related to cytotoxicity or endothelial binding.[65]

While investigators do agree that the α-galactosyl oligosaccharide residue on porcine endothelium are recognized by XNA,[66,67,68,69,70] there is still some controversy regarding the antibody isotype and exact glycoprotein structure.[40] Galili identified the XNA in human serum as IgG class, by demonstrating binding in an immunoadsorption column containing the α-Gal residue. This IgG XNA was shown to bind to porcine endothelium and epithelium.[54] Other investigators have found that XNA were: class IgM only;[71,72] IgM and IgG_2;[73] and IgM, IgG, as well as IgA.[74] While both IgM and IgG bind to porcine endothelial cells, IgG alone is not sufficient to initiate complement-mediated cytotoxicity: thus, most cytotoxic anti-porcine XNA are probably of the IgM class.[75,76] The predominant influence of IgM, compared to IgG, in eliciting HAR has been established by other groups as well.[77] More recently, the role of IgG anti-porcine endothelial antibodies in chronic graft rejection,[78] has been postulated, via a mechanism of antibody-dependent cell cytotoxicity.[54,56,79,80]

Just as the definitive role of each immunoglobulin class remains unconfirmed, the precise glyoprotein structure is also, as yet, undetermined. Platt et al. found that XNA bound to the gp 115/135 complex, a triad composed of 115 kDa, 125 kDA, and 135 kDa glycoproteins from porcine aortic endothelial cells.[71,81] These glycoproteins are homologous to human integrins,[82] which contribute to endothelial integrity: gp115 is β3 integrin, gp125 is αIIb integrin, and gp135 is α2 integrin.[83] XNA were noted to bind oligosaccharides, and not to the polypeptide core of the glycoprotein. When the β-gal subterminal residues were cleaved enzymatically, the XNA failed to bind. Further, this group has determined that a terminal component of the gp 115/135 complex is an α-Gal structure.[81] Others have shown that IgG-class XNA target different endothelial cell components. These antigens are of the molecular masses 75 kDa, 110 kDa, 180 kDa, and 210 kDa.[84]

Natural antibodies directed against porcine endothelial cells may be elaborated by a unique population of B-cells.[85] There are a number of conjectures regarding the

stimulus to produce natural antibodies. One possibility is that infection caused by an environmental pathogen may initiate natural antibody production. However, some natural antibodies are known to be present even in neonatal animals, free from any source of infection.[85] Self-antigens interacting with fetal or neonatal B-cells may be another way that natural antibodies are generated. B-cells may be produced by fetal tissues, resulting in B-1 B-cells, or adult bone marrow, yielding B-2, or conventional B-cells. B-1 precursor cells that are stimulated by antigens become B-1 cells that make natural antibodies, and persist into adult life. On the other hand, B-2 precursors respond to antigens by becoming tolerant to the antigen - either by anergy or by apoptosis.[86]

Because XNA are polyreactive - that is, they bind with more than one ligand - it is possible to inhibit XNA from binding to porcine endothelium.[82] This has allowed investigators to experiment with a strategy employing immunoadsorption of the XNA by exposing the plasma to competitive haptens.[87] A similar technique has been attempted with blood group trisaccharides, in order to allow renal allograft against the ABO barrier.[88,89,90]

Although the α-Gal structure appears to play a pivotal role in XNA function, there are other residues or epitopes that may be involved. Platt's group also described elimination of gp 115/135 reactivity to endothelial cells by adsorbing with red or white blood cells.[71] Porcine platelets bear gp115/135, but neither porcine red cells nor lymphocytes express this complex. This suggests that cross-reacting epitopes exist on these cells, perhaps representing the binding to an identical oligosaccharide substitution on a different protein or lipid. It has recently been reported that following the elimination of human serum cytotoxicity to porcine endothelial cells with immunoadsorption based on an inactivated carbohydrate-moiety, there were persistent bands on Western blot analysis.[91] These investigators concluded that this may be due to the presence of other xenoantigens in human serum. In addition, this study suggests

that it may not be necessary to deplete all xenoantibodies in order to prevent cytotoxicity. To date, neither the true clinical significance of other epitopes, nor the amount of XNA depletion required to prevent rejection, has been fully elucidated.

Other xenoepitopes that may be involved in xenograft rejection are likely related to the basic α-Gal moiety. Rydberg and colleagues reported that XNA may be directed against Galα1-4Gal (P^k antigen), as well as A and H-type antigens.[92] It has yet to be determined whether other XNA and epitopes are potent effectors of HAR or a delayed xenogeneic response.

Concordant xenotransplantation may not require XNA removal to ensure early graft survival - in fact, such antibodies may not even be demonstrable.[39] There is evidence, however, to suggest that XNA may develop following the transplant, and then proceed to initiate rejection.[93]

The Role of Complement in Xenograft Rejection

Xenograft destruction is mediated by stimulation of the complement system via three possible mechanisms: alternative pathway, classical pathway activation,[94,95] and by the failure of natural complement inhibitors.[96] The pathway stimulated depends on the specific species combination (Appendix 2: Table 4). From a clinical perspective, the fact that porcine organs only appear to suffer from the effects of natural antibody induction of complement[97,98,99] may be important in developing techniques to attenuate HAR (see "Overcoming Hyperacute Rejection" below). It is noteworthy that *in vivo* studies have found that complement in the absence of XNA does not cause HAR.[99] Most recently, it has been recognized that IgA stimulation activates the alternative pathway

in discordant xenograft rejection.[100],[101] Prior to examining strategies to alter the complement system, a brief review of the relevant complement pathways is appropriate.[102]

The classical pathway entails antibody binding to a cell, which initiates the cascade. Two Fc fragments from an antibody serve as the C1 receptor. C1 is the first component of the cascade. Once C1 is bound, cleavage of this molecule occurs, and the resultant portion activates C4. Next, C2 binds to C4, and this complex subsequently binds to C3. Conversely, the alternative pathway is independent of antibody induction. C3 is activated and stabilized by Factors B, D, and properdin. Thus, serum protein C3, which is generally present in the dormant state, serves as the major regulatory protein. Following proteolytic cleavage, C3 is activated and the destructive effector mechanisms of complement are initiated.

The pathway for complement-induced damage is similar for both the classical and alternative pathways: C3 binds with C5, resulting in the membrane attack complex (MAC, C5b-9) binding. The attack complex causes a pore in the cell membrane, and cell lysis results on an osmotic basis. The MAC mediates numerous events,[103] including: von Willebrand factor (vWF) secretion;[104] exposure of binding sites for factor Va and subsequent increased prothrombinase activity;[105] cell surface expression of P-selectin, a platelet and granulocyte adhesion molecule;[104] and procoagulation.[106]

The third mechanism of complement activation is apparently unique to xenografts. Normally, natural complement inhibitors on the endothelial cell surface serve to protect cells from lysis. These proteins, often referred to as regulators of complement activation (RCA), include decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and CD59 (homologous restriction factor, HRF).[107] These elements are known to function in a species-specific manner,[108] and thus may be critical in xenotransplantation.[109]

Endothelial Cell Activation

HAR has been described as a sequela of the loss of endothelial cell (EC) function.[82] Even if XNA depletion may prevent HAR from occurring, Delayed Xenograft Rejection (DXR, also referred to as acute vascular xenograft rejection) may ensue 2-5 days following xenotransplantation.[110] Bach and colleagues have argued that while elimination of XNA and complement may result in xenograft survival beyond the HAR phase, innate immune mechanisms will still activate EC of the grafted organ.[111,112]

EC are the target of the immune reaction in xenotransplantation, and activation of these cells will result in rejection, due to a loss of barrier and anticoagulant properties.[82] Complement-induced endothelial injury results in stimulation of EC, and XNA can specifically bind to porcine endothelium, independently activating EC.[113] It has been shown that it is the concentration of XNA - specifically IgM class - and not complement activity that determines ECA, as measured by heparan sulfate release assay.[114,115] Natural killer (NK) cells from human serum are also capable of activating and lysing xenograft EC[116] - this effect is augmented by the addition of IgG.[117] Xenograft rejection, therefore, is based on the response of EC to various stimulatory processes,[115,118,119] and studies have increasingly attributed failure of organ function to cellular-level events.[120]

The original description of the EC response identified two distinct phases - stimulation and activation.[121] The nomenclature has evolved, and investigators now classify the HAR phase as a consequence of Type I endothelial cell activation (ECA), while Type II ECA constitutes the processes that occur during DXR.[111]

Under basal conditions, EC provide a barrier function, in addition to preventing platelet aggregation from occurring. Platelet aggregation inhibitory mechanisms include prostacyclin production, nitric oxide generation, ecto-ADPases,[111] thrombomodulin expression, and heparan sulfate proteoglycan.[82] Once an inflammatory response is

evoked, the anticoagulant endothelium converts to a procoagulant surface.[122,123] It has been deduced that once EC become activated, a loss of baseline ecto-ADPase activity will allow platelet-derived ADP accumulation, which serves as a stimulant for platelet thrombosis.[124] Other anti-thrombotic proteins expressed on the EC surface include thrombomodulin[125], anti-thrombin III, and tissue factor pathway inhibitor. Heparan sulfate proteoglycan is also present on EC, binding superoxide dismutase, thus preventing degradation of reactive oxygen species. This is protective against oxidative damage[119] and production of thrombin. Release of heparan sulfate, then, may be an important step in the HAR response.[126]

EC integrity is regulated by certain components of the complement system.[127] Cytoskeletal and EC shape changes disrupt integrity, allowing intercellular gaps to occur. These changes take place within 10 to 20 minutes of EC exposure to XNA and complement. Once gap formation occurs, cell death is not imminent; instead, the complex C5b67 is required to mediate killing. The closure of EC gaps also requires complement - specifically, C5b-9 complexes.

The transformation of the endothelium to a procoagulant state has been summarized through six mechanisms.[111] Firstly, heparan sulfate acts as both an anchor and activator for antithrombin III. The loss of heparan sulfate will, therefore, promote coagulation. Secondly, thrombomodulin, which normally activates protein C as part of the fibrinolytic system, is also lost. Thirdly, the MAC is assembled on EC, exposing prothrombinase and fibrin production. Fourthly, tissue factor is elaborated, which binds to factor VIIa as part of the coagulation cascade. Next, plasminogen activator inhibitor is produce, preventing fibrin thrombus degradation. Finally, platelet activating factor (PAF) stimulates platelet aggregation. Another consideration, just recently reported, is that both thrombin production by EC and complement activation are required for platelet aggregation.[128]

Type I ECA occurs almost immediately following graft revascularization and is protein-synthesis independent.[129] This phase of the EC response can be summarized by the following events: alteration of normal defence mechanisms, such as ADPase, RCA, thrombomodulin, tissue factor pathway inhibitor, and heparan sulfate; retraction of the EC; P-selectin/E-selectin expression; inflammatory mediators, including C3a and C5a, histamine, PAF, thrombin, and leukotrienes; leukocyte recruitment; platelet thrombosis, and fibrin deposition.[129,130]

Once ECA occurs, the endothelium becomes procoagulant, through a variety of mechanisms.[131,132] EC retract from one another,[133] exposing subendothelial collagen and von Willebrand factor (vWF). Within a short period of time, P-selectin is expressed on EC surface,[111,134] and heparan sulfate cleavage occurs from membrane surfaces.[126] The presence of P-selectin present on EC surfaces, along with iC3b, promotes adhesion of neutrophils and monocytes to EC.[135] Platelet binding and proliferation occurs along the subendothelial matrix, through the interaction between platelet receptor gp1b and vWF. Increased P-selectin expression on platelets and EC, along with upregulation of the platelet receptor gpIIbIIIa, results in the release of inflammatory mediators. ADP accumulation follows the loss of ADPase function, and platelet aggregation occurs. As the increased propensity for thrombosis is established, further platelet recruitment proceeds. Fibrin is deposited due to fibrinogen binding with gpIIbIIIa, and the generalized loss of EC anticoagulant properties. The net result of these processes is HAR of the xenograft.

As Type I ECA occurs, tissue factor is expressed on EC and monocytes. Type II ECA is characterized by macrophage and natural killer (NK) cell recruitment. Tissue factor, lectins, abnormal thrombosis (loss of ADPase, antithrombin III, and thrombomodulin), and cytokines (interleukin-8 or IL-8, and monocyte chemoattractant protein, or MCP-1) are also involved in this process.[129,136] The culmination of these events is DXR. Upregulation of various genes occurs during Type II ECA.[137] Leukocyte

adhesion molecules (E-selectin, P-selectin, ICAM-1, VCAM-1), tissue factor (coagulation cascade initiation), and cytokines (IL-1, IL-6, IL-8, and MCP-1) are all induced. The increase in coaguability continues with depletion of ADPase, antithrombin III and thrombomodulin. Thrombosis can occur, similar to the events of Type I ECA. Cytokines participate in the activation of monocytes and NK cells. These cells participate in the inflammatory reaction. The procoagulatory and inflammatory state of the endothelium contributes to xenograft rejection.

In the past, ECA has been examined *in vitro*,[138,139,140,141,142] but increasing interest is being focused on *in vivo* models.[130] Platt and Bach underlined the value of this concept by testing human serum against porcine aortic endothelial cells.[71] In order to bridge the *in vitro* and *in vivo* work, Pober summarized the various interactions that can occur on the vascular endothelium.[143] Three main types of interactions were described: firstly, the graft endothelium can activate the host's immune system; secondly, the host T-cells may activate the graft endothelium to recruit an inflammatory reaction;[144] lastly, the host immune system can injure vascular EC. This is significant because porcine endothelial cells are prone to damage induced by human serum, due to the species-specific nature of regulatory mechanisms. In light of this, researchers are attempting genetic modulation of cell-surface events.

The concept of ECA does have a direct link to clinical transplantation. The pathophysiological features of ECA are being elucidated, and appear to correlate with the postulated events described above. It is known that rejection of cardiac xenografts is linked to ECA.[130] The labeling of EC demonstrated P-selectin and E-selectin, in addition to vWF. By 48 hours following graft revascularization, fibrin deposition, platelet microthrombi labelled with P-selectin, the presence of tissue factor, absence of thrombomodulin, and trace anti-thrombin III were detected.[130] Thus, increased knowledge of ECA may aid in the development of techniques to overcome the endothelial reaction to xenografts.

Accommodation

When XNA or complement are depleted, either DXR or accommodation occurs. Accommodation refers to the ability to withstand endothelial cell injury, whereby HAR or DXR do not occur. To induce a state of accommodation would ensure that the graft is resistant to antibodies that normally bind to the endothelium. It has also been postulated that continuous depletion may not be requisite for graft survival: Alexandre et al.[145] have reported that antibody-mediated rejection is less likely to occur after an initial antibody removal, even with subsequently elevated antibody titres. This state of immunocompetence is referred to as accommodation,[41] and it has been described in animal models of discordant xenotransplantation.[146,147,148]

Various mechanisms have been proposed to explain the development of accommodation.[82,149] Firstly, antibody-antigen interactions may be changed. This may be the result of some alteration in the antibodies that are directed against EC. For example, the antibodies may no longer fix complement, or participate in the antibody dependent cell-mediated cytotoxicity response. This is not likely the etiology of accommodation, as the addition of XNA known to initiate HAR does not revert the state of accommodation.[149] Another possibility is a decrease in the absolute XNA levels. It has also been postulated that antigen expression may be transformed. This explanation does not take into account those cases of accommodation where immunoglobulins were found deposited on the graft endothelium.[150]

The second theory involves EC modification. EC sensitivity to injury decreases, in spite of continued stimulation by antibodies or complement. This may be attributed to the actions of endotoxin, IL-2, and DAF. The endotoxin and IL-2[151] continue to activate the EC, resulting in increased DAF secretion.[152] Complement is, therefore, inhibited at the level of C3 convertase. All cases of accommodation reported *in vivo*

have involved antibody removal, and this is currently believed to be a key for the abrogation of HAR.

The final explanation is that an organ sustains damage during harvesting and reperfusion - antibody and complement depletion during this stage allows EC recovery. Subsequent antibody binding to the graft does not induce EC damage. This theory of accommodation takes into account the clinical events preceding transplantation that may contribute to organ injury.

Histopathological Features of Hyperacute Rejection

XNA recognize and bind to the graft endothelium, initiating the complement cascade, with graft failure rapidly ensuing.[153] The typical gross pathological features of HAR include rapid distention and accumulation of edema, accompanied by a congested appearance.[154,155]

Histopathological findings pathognomonic for HAR include the following: loss of the endothelial lining resulting in platelet and fibrin deposition on the exposed basement membrane; microvascular thrombosis; prominent interstitial xenograft edema due to markedly increased capillary permeability; expansive interstitial hemorrhage; extensive capillary dissolution; secondary myocyte degeneration; and possible necrosis.[154] Immunohistochemical studies reveal complement (C1q, C3, C5) and preformed antibody (IgG, IgM) deposition in hyperacutely rejected xenografts.[25,156] Properdin deposition was also detected, indicating alternative pathway activation of the complement cascade.

Others have reported IgM and classic but not alternative complement components binding to endothelial surfaces, platelet and fibrin thrombi, and neutrophil infiltration. These authors suggested that IgM is involved in HAR, and that complement fixation is necessary to initiate the coagulation cascade, platelet aggregation, and neutrophil infiltration.[97]

Findings associated with DXR, or acute vascular xenograft rejection, include interstitial edema, hemorrhage, thrombosis, and mononuclear and neutrophil infiltration.[82] The profound cellular infiltrate in tissue examined for DXR is attributed to macrophage and NK cell involvement.[130] This pattern is not unique to discordant xenografts, as acute vascular rejection is also seen in concordant xenotransplantation and allografts.[82,157]

APPROACHES TO OVERCOME HYPERACUTE REJECTION

It has been argued that the most successful stratagem to overcome xenograft rejection will involve a multi-pronged approach. Some researchers have focused on the role of complement in hyperacute xenograft rejection, while others are developing methods of immunoadsorption. Still other groups are focusing on achieving a state of immunocompetence. Briefly, three separate areas have been identified: depletion of xenoantibodies and xenoantigen modification; complement inhibition; and endothelial cell-based strategies.[118,158] The use of pharmacological immunosuppressive agents is also being examined, as these may be of benefit in XNA depletion.[159]

Xenoantibody Depletion & Xenoantigen Modification

In addition to the role of XNA in stimulating complement, the long-term consequences of antibody binding are unclear. Since the target glycoproteins are related to integrins,[82,83] which aid in the regulation of endothelial function, XNA binding may directly alter endothelial activity. XNA may be depleted, blocked, or antigens may be modified. XNA depletion is accomplished via plasmapheresis, immunosuppressive drugs, or *ex vivo* human blood perfusion through a xenogeneic organ.[118,158] XNA blockade is possible via carbohydrate-based immunoadsorptive techniques.[160,161,162,163,164] Synsorbs are used for specific carbohydrate immunoadsorption therapy. Xenoantigen modification has been attempted with

enzymatic cleavage of the α-Gal epitope or with molecular techniques to alter endothelial expression of α-Gal.

The initial experience with antibody-depletion was obtained from renal allografting across the ABO blood group barrier.[145,165] Antibody removal was accomplished via plasmapheresis[145] or intravenous infusion of specific synthetic carbohydrates to neutralize anti-A or anti-B antibodies.[90,165] Others have successfully employed extracorporeal immunoadsorptive methods.[166,167,168,169]

While plasmapheresis has been shown to be effective, antibody removal is non-specific. This may place the transplant recipient, who is already immunosuppressed with pharmacological agents, at an increased risk of acquiring infection. With the identification of the αGal(1-3)βGal(1-4) epitope,[56,57,58,60,66,67,68,74] investigators have attempted to target this site directly in order to ameliorate HAR in discordant xenografts.[162,163,164,170,171,172,173]

It has been suggested that immunoadsorption of XNA would be an important part of the treatment strategy.[88,90] Cairns and colleagues showed incomplete XNA blockade with α-Gal disaccharides and trisaccharides, leading these authors to postulate that modified trisaccharides, tetrasaccharides, and pentasaccharides may be required to achieve complete XNA inhibition.[164] This is still in the process of being formulated - the necessity for complete XNA depletion for xenograft survival has yet to be determined.

Ye and colleagues described intravenous infusion of melibiose and arabinogalactan, synthetic oligosaccharides which are structurally homologous to α-Gal, in an effort to block the binding of XNA to EC antigens.[160,161] A heterotopic porcine cardiac xenograft in a baboon survived for 12 hours with this approach. The intravenous route for this therapy is too toxic for clinical application, given the very high doses required to eliminate cytotoxicity. This group has also employed

immunoaffinity columns with immobilized Gal-type oligosaccharides to absorb serum. They have shown a significant reduction in cytotoxicity (approximately 80% less than controls).[160,161] These investigators have proposed using extracorporeal absorption of XNA through similar columns to prevent hyperacute rejection of discordant xenografts.

Other groups have employed this technique, also.[167,170,174,175,176] Leventhal and coworkers, for example, reported prolongation of discordant renal xenograft survival in a porcine-to-baboon model.[172] Two animals received immunsuppression (cyclosporine and methylprednisolone) and underwent repeated immunadsorption with antihuman Ig-Therasorb columns (antihuman IgG and IgM conjugated to sepharose). The xenografts continued to function, but the animals were sacrificed at 11 and 13 days for other reasons. Histopathology of the organs failed to reveal any signs of vascular rejection or significant cellular infiltrate. This technique of immunoadsorption appears to be more effective than mere plasmapheresis.

Although the mechanism of immunoglobulin inhibition of complement-mediated cell damage has not been established, this method has been employed by various groups. While Leventhal and colleagues[172] used anti-human Ig columns for adsorption in a plasmapheresis system, Gautreau et al. tested intravenous immunoglobulin therapy.[173] Investigators have also specifically depleted IgM by using an anti-μ monoclonal antibody.[177,178] The anti-μ (anti-IgM) antibody studies have been reported in a rat-mouse model - the efficacy of such a strategy has not been described in an *in vivo* primate model.

An alternative approach to preventing XNA from initiating HAR would be to eliminate the α-Gal epitope itself, either by enzymatic removal[179] or genetic engineering. The use of α-galactosidase to cleave α-Gal residues from EC results in 70 to 80% less XNA binding.[81] LaVecchio and coworkers reported that enzymatic cleavage of the α-Gal epitope resulted in significant cytoprotection of porcine endothelial cells; however,

endothelial cell reactivity was never completely abolished.[179] Furthermore, application of this technique to a complete organ xenograft would necessitate development of methods to prevent hypoxia and damage to the organ during the digestive process.

Some investigators have argued that since there are at least 40 different protein determinants expressed by porcine tissues that bind XNA, it is untenable to attempt to "knock out" all of the genes for all epitopes.[180] A more attainable goal might be to inactivate a single gene that codes for the α1,3-galactosyltransferase enzyme, and thus eliminate all α-Gal epitopes. The porcine α1,3-galactosyltransferase has been cloned with a cDNA library,[181] and antisense nucleotides targeted against mRNA decrease galactosyltransferase expression.[182]

Another method to eliminate galactosyltransferase has been tested in a murine model by preparing a construct that inactivates a galactosyltransferase allele by homologous recombination.[183] While there was considerable reduction in human serum XNA tissue binding in these chimeric mice, the animals developed cataracts. McKenzie has also cloned the α1,3-galactosyltransferase gene from mice and pigs,[184] and reported that knocking out the gene results in exposure of subterminal sugars, to which natural antibodies also exist. His group then carried on to develop a technique referred to as transferase dominance, whereby a substrate, in this instance, lactosamine, can accept either galactose or fucose.[185] Galactose is under the influence of α1,3-galactosyltransferase, while fucose is under the influence of H-transferase. After isolating and transfecting the H-transferase, they demonstrated that its activity dominates over that of α1,3-galactosyltransferase, and that the α-Galactosyl epitope is not constructed.[186] The α-Gal epitope is thus replaced by fucose, to which natural antibodies do not occur. This non-antigenic residue is actually the universal donor human blood group O antigen, bearing the structure α-1,2-fucosyl lactosamine, or the H epitope.

The technology to produce transgenic animals lacking the enzyme, and thus the α-Gal epitope, is not yet available for large-animal application. The construction of mice lacking α-Gal determinants, on the other hand, appears feasible. Galactosyltransferase-deficient mice would be expected to have natural antibodies against Galα1-3Galβ1-4GlcNAc-R, and should demonstrate HAR against tissues from donors expressing these carbohydrate determinants. Transgenic mice with H-transferase, and therefore very little α-Gal expression, are currently being tested by McKenzie.[184] H-transferase transgenic mice have α-Gal expression downregulated by approximately 80-90%,[187] which has encouraged the pursuit of this technology in a porcine model.

Japanese researchers have already constructed transgenic pigs that produce α(1,2)-fucosyltransferase by microinjection of the cDNA of this enzyme into pig embryos.[188] ^{51}Cr-release assay used to measure cytotoxicity found fibrocytes from the transgenic animals expressing the H antigen to be resistant to human serum. It is difficult to draw any conclusions regarding the true validity of such technologies until *in vivo* xenotransplant studies are performed, preferably in a primate model.

Complement Inhibition

Researchers have attacked the complement system from various angles.[189,190] C3 depletion induced by cobra venom factor (CVF) has been shown to result in a prolongation of porcine hearts undergoing *ex vivo* perfusion with human blood,[191] and a comparable result was noted for CVF used *in vivo*.[192] Similarly, naturally complement-deficient guinea pigs have been observed to reject rat hearts less rapidly.[193]

Other investigators have treated animals with soluble complement receptor type one (sCR1), which causes dissociation of classical and alternative pathway C3 convertases, demonstrating a delay in graft rejection.[194,195] Interestingly, at the time of graft rejection, sCR1-treated recipients were found to have reconstituted classical and alternative pathway activity. When xenograft recipients were treated with ongoing sCR1 infusion, graft survival was shown to be further prolonged, but rejection still occurred in spite of the lowered levels of complement.[196] Pathology revealed extensive IgG and IgM deposition. When XNA depletion was induced by pre-treating graft recipients with cyclophosphamide, significant prolongation in survival was noted.[197] Unfortunately, however, the animals succumbed to infectious complications. These studies further underscore the significant role that XNA appear to play in xenograft failure, and would appear to suggest that complement inhibition alone may not be sufficient for xenograft prolongation.

The major limitation to CVF and sCR1 is that these agents also interfere with the alternative pathway, which is necessary for defense against pathogens. Even more significant is that CVF bears a terminal α-Gal residue: repeated CVF treatment evokes significant synthesis of anti-α-Gal antibodies.[100] Thus, the use of a more specific agent, such as the classical pathway regulatory protein C1 inhibitor (C1 inh), may be more useful. *In vitro*, C1 inh has been found to inhibit cytotoxicity, and deposition of C4b and iC3b.[198] In this study, C1 inh also prevented endothelial cell activation, as measured by heparan sulfate release assay.

The MAC can also be targeted to prevent EC damage. Isolated xenogeneic hearts perfused with C8 depleted human serum, or C6-deficient rats used as discordant xenogeneic recipients do not demonstrate HAR.[199,200] This is because under these circumstances, the MAC was not formed. Monoclonal antibodies directed against both C5 and C8 have been found to prevent damage normally mediated through the MAC.[103] Membrane prothrombinase activity was also blocked with these agents. Porcine hearts

perfused in an *ex vivo* Langendorff model with anti-C5 monoclonal antibody treated human blood were found to have prolonged function.[20] In addition, histological examination of these hearts failed to reveal MAC deposition, or signs of HAR.

Another method of achieving complement depletion employs genetic engineering approaches to express membrane-bound human RCA on porcine endothelial cells.[96,107,109] This technique has the advantage of only exerting a local effect, thus avoiding possible systemic toxicity. In the case of unaltered porcine endothelium, human IgM binds EC with subsequent fixation of C1. C1 in turn activates C2 and C4. C4b and C2a then bind to the endothelium and form the C3 convertase. This is followed by the addition of C3b to the C4b-C2a conglomerate, forming the C5 convertase. The end result is the subsequent formation of the MAC. Porcine endothelim constructed to be transgenic for human DAF responds in a different fashion. Once human anti-αGal IgM binds EC, C1 is still fixed, activating C2 and C4. However, the C3 convertase formed is split by the DAF expressed on EC. MCP, another RCA, functions at a later step, by inactivating C5 convertase. A third RCA is CD59, and it its influence is exerted even further along the complement cascade, blocking MAC formation.[107]

Transgenic pigs expressing human CD59 were used as heart donors in xenograft experiments involving baboons recipients.[202] Unfortunately, xenograft survival was not substantially improved by using the transgenic organs. When compared histologically to unmodified porcine control hearts, the transgenic cardiac endothelium was found to have reduced deposition of C5b and MAC; however, C3 binding was not decreased. Japanese researchers have recently reported that double human RCA are more protective than single RCA molecules: the combination of HRF and DAF is more effective than DAF alone for resisting EC lysis in a bovine aortic EC model.[203] This has not yet been tested *in vivo*. A group from Cambridge employed yeast artificial chromosomes (YAC's) to create mice transgenic for multiple RCA, including human

MCP, CD59, and DAF.[204] They have also engineering transgenic pigs with this technology, with the entire human MCP gene contained within the YAC.[205]

The most successful results reported to date with the transgenic approach, came from the Cambridge group, who have engineered pigs transgenic for human DAF.[96,206,207,208,209,210] Greater than three week survival of a human DAF transgenic porcine cardiac xenograft in a Cynomolgus monkey recipient was achieved with the aid of heavy immunosuppression. The primates were sacrificed because of side effects from the immunosuppressive drug therapy, but the xenografts were still functional. Perhaps the most significant finding was the pristine histopathology of the heart, which failed to reveal significant immunoglobulin or complement deposition.[210] This success has stimulated White and colleagues to prepare for the possibility of a relatively early clinical trial with these genetically-altered organs.

The magnitude of this work cannot be overstated, and these results merit further comment. While such reports are well-received, and, indeed, hailed as a major breakthrough in the field of xenotransplantation, there are important questions and limitations that must not be overlooked. The approach of inhibiting the effects of complement with transgenic expression of human RCA on porcine endothelium has not obviated the need for a powerful immunosuppressive regime: cyclosporine A at 60 mg/kg/day was administered, which is a greater dose than normally used. In fact, the primate transplant recipients were sacrificed due to diarrhea - a side effect of the potent immunosuppression required. Interestingly, these primates were grafted with organs from porcine donors that fortuitously expressed low levels of endothelial α-Gal. Thus, this study did not examine the significance of XNA binding to the graft endothelium. Furthermore, it is noteworthy that transgenic donor heart median survival was merely 5.1 days without pharmacological immunosuppression.[206] Nevertheless, studies in the near future will strive to improve upon this complement inhibitory technology.

Presently, many researchers believe that porcine organs transgenic for human RCA may play a significant, albeit adjunctive, role in clinical xenotransplantation.

Endothelial Cell-Based Strategies

The complexity of the EC response to a xenograft has become the object of numerous experiments. In an effort to blunt the rejection response, researchers are investigating numerous potential targets in the ECA cascade. This section will briefly review a few of the approaches currently under study.

Specific cells may be one level for modification of ECA. The importance of neutrophil adhesion to the EC of the xenogeneic donor organ is well-established.[135] Prevention of neutrophil adhesion was useful in a small-animal model of discordant cardiac xenotransplantation when combined with complement inhibition.[211] NPC 15669, a new drug in the leumedin class of antiinflmmatory agents, prevents neutrophil involvement by blocking upregulation of an adhesion molecule (CD11b/CD18). The use of sR1 and NPC 15669 prolonged xenograft function, with evidence for decreased graft injury on histological examination. While this paper is flawed in its statistical analysis of xenograft survival, there is value in this work, as the authors have identified an area that requires study. The synergistic potential of attacking multiple events in ECA merits further investigation.

The role of P-selectin can also be exploited in an effort to prevent HAR. P-selectin is important for platelet and neutrophil binding. The use of antibody directed

against P-selectin has been shown to prolong xenograft survival in a rat model.[212] The addition of a PAF antagonist to the P-selectin antibody further prolonged xenograft function.[212] These agents were found to suppress platelet aggregation, neutrophil and macrophage infiltration, and decreased deposition of C3 and C5. This study emphasized the significance of PAF in increasing vascular permeability and the stimulation of complement. Furthermore, P-selectin and PAF would appear to play an important part in platelet aggregation and neutrophil recruitment.

Platelet gpIIbIIIa is a fibrinogen receptor that is integral to platelet aggregation and adhesion to subendothelial surfaces. GPI 562, a specific gpIIbIIIa antagonist, did not improve *ex vivo* cardiac function or survival, but did appear to confer some degree of protection to EC by immunohistochemistry: the GPI 562 treated group was found to have reduced white cell extravasation and platelet thrombosis.[213] The reduction in EC damage led these investigators to suggest that anti-platelet therapy may be useful to prevent DXR. However, immunohistopathological examination was reported as an average arbitrary score, and the experimental group did not differ from controls with respect to endothelial retraction. Further, neutrophil adherence and fibrin deposition appeared to have been reduced only slightly in the GP1 562 group. As with many of the other modalities being studied, the merit of platelet inhibition as it might apply to clinical xenotransplantation remains unclear.

The stimulation of platelets and subsequent thrombosis likely requires thrombin. In order to investigate this further, Robson and coworkers tested SDZ MTH 958, a thrombin inhibitor, in an isolated perfused heart model.[214] These authors reported prolonged survival and enhanced function in this *ex vivo* study. Thrombin inhibition also improved histological features. There were significant flaws in experimental design and reporting of data; however, this does represent the first study with a thrombin inhibitor, and should not be dismissed. As with the gpIIbIIIa antagonist, the value of a thrombin inhibitor requires further clarification.

The ubiquitous transcription factor NF-κB is felt to be central to gene induction in ECA.[215] The agent pyrrolidine dithiocarbamate (PDTC) inhibits NF-κB activation. Preliminary studies have shown that PDTC inhibits E-selectin, IL-8, and tissue factor.[111,216] Thus, NF-κB blockade may represent a useful modality in the modulation of EC reaction in xenotransplantation.

The Harvard group is keen on focusing on the endothelium of the xenograft for genetic modification.[216] The procoagulant environment that results following ECA may be counteracted by the use of endothelium expressing human thrombomodulin. NF-κB can also be targeted for molecular alteration. If this transcription factor is blocked, upregulation of the various genes involved in ECA may also be prevented. IκBα is a naturally-occurring inhibitor of NF-κB. The use of a recombinant IκBα adenoviral vector to transduce porcine EC suppressed gene transcription.[216] This technology may be successful for creating transgenic donors for humans.

Other novel strategies include the development of monoclonal antibodies directed against porcine vascular cell adhesion molecule (pVCAM),[217] endothelial reseeding. This approach entails enzymatic degradation of the donor endothelium with collagenase. The recipient endothelium is then used as a replacement on the graft. Initial small animal studies have been reported,[218] however, to date the utility of this method in the porcine-human situation has not been established.

Pharmacological Immunosuppressive Agents

The development and experience with various pharmacological immunosuppressive agents has allowed allotransplantation to become a successful procedure. This is particularly true of cyclosporine (CsA), which quickly became a

mainstay of the immunosuppressive regimen. To date, immunosuppressive drugs alone have been unable to provide sufficient immunosuppression to permit the survival of xenografts. However, newer agents, in combination with some of the approaches outlined above, may help to make xenotransplantation a clinical reality.

Immunosuppressive drugs - such as steroids, CsA, FK506, and antilymphocyte globulin - that primarily inhibit T-cell functions, have had limited success in prolonging xenograft survival.[219,220,221,222] Due to importance of humoral events in HAR, increasing interest has been focused on agents that inhibit B-cell activation. Most notably, brequinar, leflunomide, mycophenolate mofetil, rapamycin, and deoxyspergualin have been examined.[223,224,225]

New drugs mediate effects on the immune system by acting on various target enzymes, which normally act as catalysts for normal immune reactions. The mechanisms of immunosuppressive drugs can be organzied as follows: 1) inhibition of interleukin or cytokine synthesis; 2) interleukin or cytokine and growth factor inhibition; 3) mechanisms which are unclear.[226]

The evolution of immunosuppressive therapy has been well-reviewed.[226,227] Historically, radiation and chemical reagents were investigated as the earliest immunosuppressive agents, based on the destruction rapidly dividing cells.[227] Pharmacological immunosuppression began with 6-mercaptopurine (6-MP), a competitive purine synthesis inhibitor. The mechanism of action of 6-MP was reduction of antibody production. Azathioprine (AZA), a 6-MP derivative, was developed as a more stable replacement for 6-MP. Corticosteroids were added to these agents in an effort to provide more effective immunosuppression.

Immunosuppressive therapy further evolved as T-cells were targeted. Antilymphocyte serum employed polyclonal antibodies with strong immunosuppressive activity.[228] This advance increased the survival of grafts by T-cell inactivation.

Monoclonal antibodies (MAbs), more specific molecules directed against the T-cell receptor, were then subsequently developed. The resultant effects of MAbs include increased cytokine release, lymphoid cell activation or destruction, and the production of human anti-mouse antibodies. These antibodies are later able to interfere with the function of MAbs.

Another breakthrough in immunosuppressive therapy occurred with the development of agents that targeted cells that inhibit cytokine synthesis. T-cell activation is inhibited by blockade of the IL-2 transcription signal. IL-2 and its receptor are required for T-cell growth and proliferation. CsA was the original drug used for immunosuppression in this class.[227] CsA has been largely responsible for the prolongation of allograft survival, and thus the overall success of this procedure.[229,230,231,232] This agent exerts is effects by binding the cytoplasmic protein immunophilin CyP (cyclophilin),[233] and then attaching to calcineurin. Calcineurin is a cytoplasmic phosphatase involved with calcium dependent T-cell activation. Although CsA inhibits lymphokines and cytotoxic T-cells, it does not affect suppressor T-cell maturation.[227] CsA does have potential toxicities: nephrotoxicity remains a significant side effect of CsA therapy.[234]

Tacrolimus (FK506) is also a lymphokine synthesis inhibitor, but is purported to be up to 100 times more potent than CsA.[227] FK506 was discovered in 1984, and the first published report of immunosuppressive activity followed in 1987. By interacting with the immunophilin FK binding proteins (FKBP), FK506 impedes calcium-dependent signal transduction, via the calcineruin-calmodulin system. The expression of IL-2 and IL-7 receptors is downregulated, and cytokine transcription is inhibited - lymphocyte activation is subsequently impaired. FK506 also functions to inhibits T-cell-dependent activation of B-cells. Some centres are altering immunosuppressive regimens to incorporate FK506 - for example, tacrolimus used in cardiac transplantation instead of CsA results in comparable survival, but with fewer adverse effects.[235]

Lymphokine signal transduction is impeded by suppression of IL-2 responsiveness in a second class of drugs. Representative agents include sirolimus (rapamycin, RAPA), and leflunomide. RAPA was discovered in the mid-1970's, but the potential for use in transplantation was not explored until the late-1980's. Due to its lipophilic nature, RAPA traverses the cell membrane without difficulty, and then binds to cytoplasmic FKBPs. T-cell protein synthesis is obstructed due to the inhibition of a kinase by RAPA. Compared to CsA and FK506, RAPA is less efficient in suppressing the elaboration of cytokines. The pathways affected are calcium-independent in both T and B-cells. Immunoglobulin synthesis is inhibited by RAPA, and at high concentrations, NK cells and antibody dependent cellular cytotoxicity are suppressed. Allograft trials for renal and cardiac patients are proceeding, and this agent may have a role in xenotransplantation.

The third group of drugs inhibit DNA synthesis. Examples from this category include mycophenolate mofetil and brequinar. Leflunomide and azathioprine are also inhibitors of DNA synthesis. Mycophenolate mofetil is merely the prodrug - the active metabolite is mycophenolic acid (MPA). MPA was introduced in the immunosuppressive arena in the 1980's, although it was tested many years earlier as an antineoplastic agent. MPA is known to reversibly inhibit of inosine monophosphate dehydrogenase (IMPDH), an enzyme required for purine synthesis during the process of lymphocyte activation. Thus, MPA inhibits lymphocyte proliferation. In addition, IMPDH inhibition results in guanosine nucleotide depletion. Of particular relevance to xenotransplantation is MPA's T-cell-independent inhibition of antibody synthesis by B-cells.

Brequinar depletes intracellular pyrimidine pools through reversible inhibition of dihydroorotate dehydrogenase, an enzyme in the *de novo* pyrimidine synthesis pathway. Brequinar appears to be selective in its suppressive effects on T and B-cell proliferation

based on the amount of DNA and RNA synthesis required by these cell lines. Antibody production is therefore also inhibited by this agent.

Leflunomide is the newest agent developed for transplantation. It has been shown to antagonize cytokines, and inhibit both T and B-cells.[236] B-cells appear to be particularly prone to inhibition by leflunomide. The exact mechanism of action for this drug has not been defined. It is possible that leflunomide inhibits IL-2 receptor-associated tyrosine kinase activity or pyrimidine synthesis. Because of its long half-life, leflunomide itself will not be used clinically, but synthetic analogues may be employed.

The fourth group of immunosuppressants to consider are inhibitors of antigen processing, presentation, and reception. Steroids bind to intranuclear receptors, displacing heat-shock proteins. The result is interference with antigen transportation in the antigen presenting cell, as well as obstruction of transcriptional events. MAbs, for example, OKT3, and 15-deoxyspergualin (DSG) function intercede in antigen presentation or T-cell activation. The MAbs employed in clinical transplantation are directed against the T-cell surface or cell surface receptors on antigen presenting cells. Signal transduction is suppressed by the impairment of antigen recognition that results from MAb therapy. DSG binds heat-shock proteins 70 and 90, which participate in binding and intracellular transport of antigenic peptides wihin the antigen presenting cell. DSG may also hamper communication between heat-shock proteins that are involved in glucocorticoid receptor stimulation.

The role for immunosuppressive therapy in xenotransplantation is still evolving. White's experience with transgenic organs would appear to indicate that these drugs will still be essential for successful xenografting in the foreseeable future. This is especially true with respect to control of XNA synthesis, which may be possible with RAPA, MPA, and leflunomide. With further refinements of some of the newer drugs, the synergistic effect of pharmacological agents with complement inhibition, XNA

depletion/blockade, and EC surface modification, the introduction of clinical xenotransplantation may be hastened.

HYPOTHESIS

The multitude of strategies currently being explored would suggest that multiple events must be targeted to consistently prevent HAR. Complement inhibition may be essential to ensure xenograft survival, but studies focusing on complement have failed to assess the effect of persistent XNA upon the endothelium. While technologically attractive, genetic engineering requires chance recombinant events during cell replication, as well as the appropriate breeding of progeny. Such manipulations are time consuming and labor-intensive. Since XNA are initiators of a cascade of responses, including the complement system, direct depletion or blockade of antibodies may be the most effective target. This could be easily accomplished with an appropriate compound, and elimination of XNA has the added advantage of removing a potential endothelial-cell stimulant. For these reasons, XNA are a most appropriate point of attack.

One specific approach to overcome HAR is XNA adsorption by synthetic carbohydrates, which contain moities to which the XNA are directed. Synsorbs are immobilized carbohydrates bound to a solid support matrix. This product is more suitable for antibody removal compared to plasmapheresis or polyclonal antibodies against human immunoglobulins,[172] since Synsorbs specifically eliminate only the antibody of interest. The Synsorbs which are most efficacious in reducing the cytotoxicity of human serum to porcine endothelial cells have been identified, and bear the chemical structure αGal(1-3)βGal(1-4)βGlc-R.[66,74,91] Synsorbs have been successfully employed for *in vivo* studies of cardiac allografting across the ABO blood group.[90] Clinically, Synsorb immunoadsorption has permitted ABO incompatible renal allografting.[168,237,238] Previous *in vitro* studies have shown Synsorbs to be potentially

useful in xenotransplantation, [66,74, 91, 162,239] but *ex vivo* and *in vivo* experiments have not been reported.

In view of the above, a study was embarked on to test the efficacy of Synsorb 90 for the prolongation of survival of *ex vivo* neonatal porcine hearts perfused with human blood products.

MATERIALS AND METHODS

Experimental Animals

Neonatal piglets (age 1 to 2 days) were obtained through Health Sciences Lab Animal Services, University of Alberta. Blood group antibodies may cross-react with the xenogeneic epitope, since the chemical structure of the α-Gal epitope consists of a non-fucosylated form of a B blood group antigen. In order to prevent this, all human plasma used was of blood group B, and all piglets were A negative. Piglets were initially blood typed with a monoclonal antibody (generously provided by the Canadian Red Cross, Blood Services, Edmonton, AB, Canada) by a tile technique, which was later confirmed using a tube technique. All animals received care in compliance with the guidelines governing handling of laboratory animals (Animal Ethics Committee, University of Alberta, in accordance with the Canadian Council on Animal Care guidelines for the care and use of experimental animals).

Human Blood Products

All experiments employed group B fresh frozen plasma collected into citrate-phosphate-dextrose (CP2D). All human blood products used in this study were generous gifts of the Canadian Red Cross Society (Blood Services, Edmonton, AB). All studies were conducted with pooled plasma from a minimum of three individual donors.

Porcine Blood Products

Porcine whole blood was obtained from four adult donors from a local abattoir. The blood was collected in a plastic vessel containing heparin sodium (100 units/mL) at a concentration of 1 unit/mL of whole blood, and centrifuged at 7000x g for 10 minutes to separate the plasma component. Plasma samples were pooled and immediately frozen and stored at -70°C, thus obtaining fresh frozen pooled porcine plasma.

Immunoadsorption

Prior to performing immunoadsorption, plasma samples were collected and stored in 1000 μL plastic centrifuge tubes for future enzyme linked immunosorbent assay (ELISA), and C3, C4 complement levels (as described below). Chromosorb P, the solid support matrix for the immobilized carbohydrates without the actual antigenic epitope, and Synsorb 90 were obtained from the Alberta Research Council (Edmonton, Alberta, Canada). The immunoadsorption was performed using a batch-method, with 0.2 mg of Synsorb 90 or Chromosorb P per mL of plasma. The plasma and immunoadsorptive materials were agitated overnight at 4°C. Following a twelve hour period of agitation, the sample was centrifuged at 7000x g for 15 minutes, with the resulting plasma portion removed for isolated heart perfusion. Untreated plasma samples were agitated overnight in a similar fashion without immunoadsorptive substances.

Perfusate Electrolyte Optimization

Once the perfusate solution was recovered by centrifugation from the immunoadsorptive agent, the sample was anti-coagulated with heparin sodium (4 units/mL of plasma). Electrolyte and blood gas samples were taken and measured via i-STAT (i-STAT Corporation, Princeton, NJ) or Nova analyzer (Nova Biomedical, Boston, MA) using ion-selective electrode technology. The ionized calcium was corrected to at least 0.9 mmol/L by adding 10% w/v $CaCl_2$. Sodium bicarbonate (8.4% w/v), and potassium (40mEq/20mL KCl) were adjusted as necessary in an effort to maintain HCO_3^- 18-24 mmol/L and K^+ 3.5-6.0 mmol/L. Repeat measurements were used to confirm electrolyte and blood gas balance. Plasma samples were collected for antibody determination and complement measurements.

Surgical Procedure

All animals received 100% oxygen via mask during the harvesting procedure. Piglets were anesthetized with Halothane, induced at 3.0-3.5% (dose-titrated until the animals were unresponsive to painful stimulus), and maintained at 2.5-3.0%. The animals were prepared and draped in the standard fashion, and a median sternotomy incision was made. The pericardial sac was entered, and the intrapericardial portion of the inferior vena cava (IVC) was isolated with a 3-0 silk ligature. One mL of blood was drawn for confirmation of blood type, followed by 100 units/kg of heparin being injected into the IVC. The superior vena cava (SVC) was isolated with a 3-0 silk tie. The IVC was tied off, and the SVC was vented distal to the untied silk ligature. As back bleeding from the SVC occurred, the aorta was cross-clamped at the level of the innominate artery. Approximately 1.7 mL/kg of standard cold cardioplegia was injected into the aortic root, proximal to the cross-clamp. Upon arrest, ice cold saline was immediately

applied to the surface of the heart. The cold saline was periodically refreshed throughout the remainder of the surgical procedure. The SVC ligature was tied, and the aorta and pulmonary artery were divided. The pulmonary veins were then gang-tied, divided distally, and the heart was excised. The heart was placed directly into an ice cold saline bath, and cold cardioplegia was re-administered (1.7 mL/kg). The aorta and pulmonary artery were dissected free, and trimmed as necessary. The organ was then dried and weighed. Prior to attaching the heart to the perfusion apparatus, cold cardioplegia was injected via the aortic root, to flush the coronary arteries. The aortic root was cannulated, and the cannula was secured with a 3-0 silk ligature.

Isolated Perfused Heart Apparatus

The perfusion circuit employed is diagrammed in Appendix 3 (Figure 1). The system consisted of a glass reservoir, peristaltic pump, glass heat-exchanger and oxygenator. The oxygenator was fixed at a height of 80 cm above the heart throughout the experiment. All glassware was water-jacketed, and custom-built to our specifications (Technical Resource Group, Scientific Glassblowing Services, University of Alberta, Canada). The system was designed on a manifold to support three experiments simultaneously by sharing carbogen (95% O_2, 5% CO_2) supply and warm water re-circulating throughout the jacketed glassware. All three pumps were peristaltic in nature (Cole-Parmer, Niles, IL, USA; Sarns, Ann Arbor, MI; and Piper, Dungey, Mississauga, ON, Canada). All perfusion lines were three-eighths inch outer diameter and one-quarter inch internal diameter silastic tubing. In-line filters (Sartorius, obtained from Cole-Parmer) with 60 μm pre-filter and 20 μm filters (Millipore, Bedford, MA, USA) were also placed. Temperature monitoring was achieved by using copper-

constantan thermocouples (type T), with thermocouple readers (Cole-Parmer). Flowmeters by Cole-Parmer were used to adjust gas flow rates.

Isolated Heart Perfusion

After the acid-base and electrolyte status was optimized, as described above, the system was primed with 150 mL of the perfusate. Once the aortic root was cannulated, the heart was incorporated into the circuit, and perfusion commenced. At fixed intervals, acid-base and electrolyte determinations were made. Any abnormalities detected were immediately corrected by adding the appropriate electrolytes or adjusting gas flow (as above). The heart rate and coronary perfusion were also directly measured at fixed time points (0, 15, 30, 60, 120, 180, and 240 minutes). The experiment was terminated at 240 minutes, or when the organ failed. Organ failure was defined as asystole, ventricular fibrillation, or ventricular asystole. At the conclusion of the experiment, the organ was dried and weighed, and sections were snap frozen in liquid nitrogen. The remainder of the tissue was fixed in buffered formalin (10%). Plasma samples were once again collected for *in vitro* assays.

Histopathology and Immunohistochemistry

All specimens were fixed by immersion in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin for routine evaluation, and with immunohistochemical markers (Sigma Chemical Co., Mississauga, Ontario) for

localization of IgA, IgM, and IgG. Staining intensity was scored semi-quantitatively by two independent examiners on a scale of 0-4+ in a blinded fashion.

Measurement of Antibody

Total antibody (human IgG and IgM) were measured by using a direct ELISA. Ninety-six well EIA plates (NUNC-immuno plates, Roskilde, Denmark) were coated with 1 μg/well of 90-bovine serum albumin (BSA) antigen in coating buffer (0.05 M sodium carbonate/sodium bicarbonate). The plates were incubated at 4°C overnight. The following morning, the contents of the wells were removed by inverting. The plates were washed for 5 minutes with 200 μL/well of wash-buffer (PBS + 0.05% v/v Tween). The wash buffer was removed by inverting and blotting. 200 μL of blocking buffer (PBS + 1% w/v BSA) was added to each well, and incubated for one hour at room temperature. The blocking buffer was discarded in the same manner as described previously. Plasma diluted at 1:10 (100 μL) was added to each well. The plate was then incubated at room temperature for 90 minutes. Following the incubation, the plates were washed three times for 5 minutes with 200 μL/well of wash buffer. The excess buffer was discarded. One hundred μL of a 1:1500 dilution of secondary antibody (anti-human polyvalent immunoglobulin [α, γ, and μ specific] alkaline phosphatase conjugate) (Sigma) in dilution buffer (PBS + 0.05% v/v Tween) was added to each well, and incubated for 90 minutes at room temperature. At the conclusion of the incubation period, the plates were washed with 200 μL of wash-buffer (5x5 minutes). The excess buffer was then removed. Para-nitrophenyl phosphate disodium (Sigma) enzyme substrate solution was prepared in 10% w/v diethanolamine buffer (pH=9.8). The substrate was added to each well (100 μL) and incubated at room temperature, in the dark. The O.D. was read at 405 nm with a Titertek Multiscan Plus at 30 and 60 minutes.

MTT Cytotoxicity Assay

These studies were performed using a method adapted from Mossman's original description,[240] and subsequently modified by Tuso et al.[241] In brief, LLC-PK1 cells, a renal tubular epithelial cell line (American Type Culture Collection, Rockville, MD), were grown to confluence in 96-well plates (Intermed Nunc, Denmark). The growth media was removed by inverting and blotting, and 100μL of human plasma samples in a 1:4 dilution in phenol-free M199 were added to each well. Following a 90 minute incubation at 37°C, the plasma samples were removed by inverting and blotting. 20μL of rabbit complement (Pel Freeze, Brown Deer, WI) was added to each well. The plates were then incubated at 37°C for one hour. After the incubation, the complement was removed as before, and the cells were washed with phenol red-free M199. 50μL of MTT (3-4,5-dimethyldiazol-2-yl-2,5-diphenyl tetrazolium bromide) (Sigma, St. Louis, MO) in a 1 g/L solution in medium was added to all wells. The cells were then incubated for 3 hours at 37°C, after which time the plates were centrifuged for 5 minutes at 1000x g. At this point, the MTT was removed by inverting and blotting, and 100μL of propanol was added to all wells in order to dissolve the formazan crystals formed by the living cells. Once complete dissolution was achieved, the plates were read at 570 nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA).

All MTT cytotoxicity assays were performed in quadruplicate with a positive control consisting of cells treated with 3% (w/v) Triton-X 100. Pig serum incubated with complement was used as a negative control, and cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = [-(\text{O.D. sample} / \text{O.D. negative control}) + 1] \times 100\%$$

Due to the variability in serum cytotoxicity in the general population, an internal control was established: sample cytotoxicity was determined as a baseline cytotoxicity

upon thawing of the plasma (pre-adsorption cytotoxicity), and compared to the cytotoxicity measured following immunoadsorption, immediately prior to *ex vivo* perfusion. The difference between the pre-adsorption and pre-perfusion samples was referred to as the percent change in cytotoxicity. The untreated human plasma control was handled in an identical fashion, with the pre-perfusion sample being drawn after the 12 hour agitation at 4°C without any immunoadsorptive agents.

Complement Levels

C3 and C4 determinations were made using a nephilometric procedure on the Hitachi 911 system (Boehringer Manneheim Inc., Dorval, Que, Canada) in the University of Alberta Hospitals clinical laboratory. Reagents were obtained from Boehringer Mannehiem Inc.

Statistical Analysis

Statistical assessment of immunohistochemical staining was carried out by Kruskal-Wallis rank sum test. A p value of less than 0.05 was considered significant. All quantitative data were recorded in spreadsheet form (Excel 7.0, Microsoft Corporation, Roselle, IL). Data analysis was performed by analysis of variance (ANOVA) or regression models (Pearson product moment correlation coefficient, stepwise regression), where appropriate, by SigmaStat 2.0 (Jandel Scientific) statistical software package.

RESULTS

Four arms were employed in this study, each with a different perfusate for the heart: (1) neonatal porcine hearts perfused with pooled porcine plasma (n=5); (2) neonatal porcine hearts perfused with untreated human plasma (n=7); (3) neonatal porcine hearts perfused with Chromosorb P adsorbed human plasma (n=5); (4) neonatal porcine hearts perfused with Synsorb 90 adsorbed human plasma (n=5). The pooled porcine plasma perfusate served as the basis for assessment of survival and function of the isolated perfused hearts, while the untreated human plasma was used as the control to demonstrate hyperacute rejection. Chromosorb P adsorption of plasma was employed as a further control to compare with Synsorb 90 treatment of plasma for antibody removal, and *ex vivo* survival and function.

Survival of Isolated Perfused Hearts

Individual and mean survival times of Langendorff perfused neonatal porcine hearts with various perfusates are shown in Appendix 4 (Figures 2 and 3, respectively). Hearts perfused with pooled porcine plasma survived for 240 minutes (n=5), at which time the experiments were terminated. Perfusion with untreated human plasma resulted in a mean survival of 124.9 minutes (n=5). Human plasma adsorbed with Chromosorb P had a survival of 67.4 minutes (n=5), while Synsorb 90 immunoadsorption prolonged organ survival to 240 minutes in all cases (n=5). Examination of these data revealed that this difference in survival times among groups was significant ($p<0.005$) by ANOVA. Dunn's method was used for post hoc

comparisons, and Synsorb 90 was found to differ from Chromosorb P, the true negative control ($p<0.05$).

The heart rate was measured at fixed intervals throughout the course of the experiment in an effort to characterize the function of each group. The mean heart rates are shown in Appendix 4 (Figure 4). Hearts perfused with pooled porcine plasma demonstrated the best and most consistent rate, with a mean of 132 beats per minute (bpm). Towards the end of the experiment, there appeared to be a decline in the rate of porcine plasma hearts. This was due to a single study where the organ became profoundly bradycardic over the final 20 minutes. After an initial mean rate of 154 bpm, Synsorb 90 perfusion resulted in a mean heart rate of 105 bpm. This would appear to be relatively bradycardic for a neonatal heart: nevertheless, this is superior to the Chromosorb P and untreated control groups, both with a mean rate of 97 bpm.

The mass of the heart was recorded prior to and following perfusion, and the weight change was used as a measure of edema. The amount of edema accumulated during the *ex vivo* perfusion was calculated relative to the duration of heart survival, which reflected the vigor of rejection (Appendix 4: Figure 5). Since plasma was being used as a perfusate in all cases, it was felt that any edema due to colloid deficiency would be minimal. Synsorb 90 and porcine plasma perfused organs gained less weight (0.31 ± 0.05 % and 0.16 ± 0.04 % respectively) than untreated plasma and Chromosorb P (2.14 ± 3.53 % and 2.95 ± 1.89 % respectively). Kruskal-Wallis one way rank sum ANOVA confirmed statistical significance amongst groups ($p<0.005$), and post hoc comparison by Dunn's method revealed a difference between Synsorb 90 and untreated human plasma ($p<0.01$).

Antibody Levels Following Immunoadsorption

Determinations by direct ELISA revealed that Synsorb 90 immunoadsorption significantly depleted anti-porcine human IgG and IgM compared to Chromosorb P adsorbed samples and untreated human plasma ($p<0.001$ by ANOVA) (Appendix 4: Figure 6). Three of the samples that underwent Synsorb 90 adsorption demonstrated at least a 98% reduction in specific immunoglobulin levels. The other two samples originated from a pool with a substantially lower baseline level of anti-pig natural antibodies, with an optical density (O.D.) of 0.090 versus 0.520 and 0.529 for the other pools. In spite of this natural individual variation in baseline XNA titres, Synsorb 90 treatment of plasma resulted in a statistically significant depletion of immunoglobulins ($p<0.05$ by Tukey test), with a mean reduction of 75.97 ± 30.4% (mean ± standard deviation) (Appendix 4: Figure 6).

Human Serum Cytotoxicity

In addition to its efficiency in XNA removal, Synsorb 90 immunoadsorption was also the most efficacious method of cytotoxicity depletion, when compared with controls (Appendix 4: Figure 7). This relationship was not found to be statistically significant ($p>0.05$) by ANOVA, however. It is possible that with additional subjects that this tendency would have approached a level of statistical significance.

Complement Determination

C3 and C4 levels were performed to confirm the presence of complement (Appendix 4: Table 5). Laboratory analysis revealed that C3 and C4 were detectable in significant quantities, even following the immunoadsorption procedure.

Histopathology and Immunohistochemistry

Morphological assessment for the *ex vivo* hearts is summarized in Appendix 4 (Table 6). Antibody staining, localized along the endothelium of large and small blood vessels, was most intense for IgM followed by IgA and IgG. This pattern was seen between and within the various treatment groups, the one exception being hearts perfused with porcine plasma for which staining of all three antibodies was absent (as expected). IgA and IgM staining was significantly less ($p<0.02$ by post hoc comparison, following Kruskal-Wallis ANOVA) in Synsorb 90 perfused hearts, compared to the Chromosorb P group, the negative control. A similar trend was also noted with IgG. Sections stained with hematoxylin and eosin revealed a normal appearance for muscle fibers, interstitium and blood vessels in all heart specimens, regardless of treatment protocol.

DISCUSSION

Xenograft rejection is a continuum, divided into three phases: initially, HAR results in a violent response; DXR, the mechanism for graft failure days to weeks after xenotransplantation; and chronic rejection, likely T-cell mediated.[129] In order for xenotransplantation to become a clinical reality, hyperacute rejection must first be eliminated. Besides XNA depletion, various other modalities are currently under investigation, ranging from complement inhibition via the generation of transgenic donors,[207,208,209] to genetic alteration of the xenograft's endothelium.[216] In addition, various experimental pharmacological [225] and molecular therapies [242] are being studied. Removal of XNA may be critical for the xenograft recipient, however, as there is a correlation between α-Gal level and the amount of cytotoxicity to porcine epithelial cells.[65] Additionally, clinical experience has demonstrated a rapid return of XNA titres in a XNA-depleted patient following transfusion of human blood products,[26] which likely contributed to graft failure.

It is likely that XNA removal would represent an important adjunct to other therapies directed against hyperacute rejection, as the long term consequences of persistent XNA levels have yet to be fully elucidated. It has been proposed that IgG directed against the porcine endothelium may play an important role in the chronic rejection of xenografts.[78] In addition, XNA depletion may contribute towards achieving accommodation.[118] Thus, XNA depletion remains an area of interest for investigators.

Methods for XNA reduction employed clinically include plasmapheresis, organ perfusion,[243] pharmacological immunosuppression, and immunoadsorption. Plasmapheresis is a powerful, but non-selective modality for removing antibodies, which may place the graft recipient at an increased risk of contracting infection. Furthermore,

the coagulation cascade may be compromised. Organ perfusion is more selective in that XNA are removed from the recipient by virtue of exposing the antibodies directly to the xenoantigens of interest. This method, however, requires a significant circulating blood volume. In addition, clotting factors and complement depletion may occur. A number of new immunosuppressive drugs which inhibit antibody synthesis, such as RAPA, mycophenolate mofetil, and leflunomide have potential for use in xenotransplantation. However, individuals would have to be pre-dosed months in advance, to allow sufficient depletion of XNA to occur. Thus, specific immunoadsorption of XNA may represent the most practical alternative.

It has been previously shown that Synsorbs were effective for *in vivo* depletion of natural antibodies, in the case of cardiac allografting across the ABO blood group barrier.[88,90] This study employed an injectable Synsorb preparation. When these investigators attempted to eliminate XNA with other carbohydrates bearing terminal non-reducing α-galactose, significant toxicity resulted.[161] Leventhal et al recently reported significant antibody depletion using polyclonal antibodies against human immunoglobulins.[172] This strategy relied on a compound which is less specific in antibody removal, possibly placing the immunosuppressed xenograft recipient at increased risk. Based on these studies, Synsorb 90 appears to be more effective and less toxic than other carbohydrates and compounds for the purposes of immunoadsorption. Furthermore, the safest approach may involve extracorporeal immunoadsorption with Synsorb 90.

It has been suggested by other investigators that AB blood should be used for investigation of HAR, in order to prevent cross-reactivity of the anti-B antibodies with the αGal(1-3)βGal(1-4) epitope.[244] This is because the blood group B antigen also contains the linear B structure. Blood group AB, however, is generally in short supply in blood banks. For this reason, only B plasma and A- (O) piglets were employed in this

series of experiments, thus avoiding both blood group incompatibilities and cross-reaction of blood group antibodies present in the plasma with the α-Gal epitope.

The decision to employ a non-working heart was the direct result of the logistical limitation of obtaining adequate volumes of human blood products. The initial assessment of a beating non-working heart model revealed that this perfusion circuit required one-half of the volume of perfusate, as compared to a working heart model. Furthermore, the isolated perfused heart was one of various modalities used to corroborate the efficacy of Synsorb 90 XNA depletion: that is, the success of the experiment did not purely rely upon physiological parameters of cardiac function.

It was recently reported that multiple plasmapheresis treatments reduced XNA to low levels, while immunoadsorption was less satisfactory: protein A immunoadsorption did not remove IgM, and soluble oligosaccharides with terminal α-Gal epitopes resulted in only a 40-60% reduction in XNA.[245] Others have tested newly synthesized α-Gal oligosaccharides; however, this product was only 25-60% and 50-70% effective for reducing anti-αGal IgM, and IgG, respectively.[163] The results of batch method immunoadsorption with Synsorb 90 reported here suggest that Synsorbs are more efficacious for XNA inhibition compared to other compounds. ELISA results revealed that Synsorb 90 was able to successfully deplete greater than 98% of XNA in three of out five samples. The other two cases had relatively low baseline XNA titres even prior to immunoadsorption, which would account for an overall less effective XNA depletion. The mean values for XNA removal are depicted in Figure 6 (Appendix 4).

Previous investigators have reported that HAR was not consistently demonstrable in *ex vivo* porcine cardiac perfusion with human blood, but the use of human plasma did result in rapid organ failure.[246] The use of whole blood necessitates hemodilution for *ex vivo* perfusion, which also dilutes the overall XNA load exposed to the organ. For this reason, human plasma was employed as a perfusate in this study,

and accordingly compared to porcine plasma. Human plasma controls did cause rapid deterioration in organ function, confirming the occurrence of HAR.

Another consideration in the use of fresh frozen plasma was the presence of complement. There have been reports of complement depletion during *ex vivo* perfusion.[213] Plasma samples were assayed and confirmed what would appear to be adequate levels of C3 and C4 to mediate HAR (Appendix 4: Table 5). Even though plasma samples may have complement levels outside of the normal reference range, it was felt that there was sufficient complement to cause rejection in this model. This would explain the rapid organ failure noted in control groups.

The *ex vivo* emulation of porcine-to-man cardiac xenotransplantation has reproduced the prolongation of graft survival following XNA depletion (Appendix 4: Figure 3). Synsorb 90 immunoadsorbed human plasma resulted in the heart functioning for 240 minutes in all instances - identical to the porcine plasma controls. This was in contrast to the earlier deterioration and failure of untreated and Chromosorb P adsorbed human plasma. ANOVA of survival times verified the statistical significance of this observation ($p<0.001$). Two hearts in the untreated human plasma group deserve comment: these organs were able to function for 240 minutes. This may be due to individual variations and susceptibilities of the piglets, as up to 10% may have low expression of gp 115/135;[247] as well, there are natural population variations in XNA levels and cytotoxicity of human plasma. Overall, hearts perfused with untreated human plasma demonstrated poorer function, compared to the Synsorb 90 group.

Cardiac performance was also mildly improved in the Synsorb 90 group (Appendix 4: Figure 4). While hearts perfused with porcine plasma demonstrated the most consistent function, the Synsorb 90 group were observed to have adequate heart rates - better than the untreated and Chromosorb P groups. There was also less fluctuation in these values between hearts in the Synsorb 90 arm of the study, when

contrasted with the human plasma controls. In an attempt to assess the vigor of the rejection response, weight change over the course of the perfusion experiments was used to quantify the accumulation of edema (Appendix 4: Figure 5). Analysis of these data revealed a significant difference between groups ($p<0.005$): Synsorb 90 and porcine plasma perfusates gain markedly less weight than Chromosorb P and untreated human plasma. It is possible, however, that the majority of edema accumulated early, and that the prolongation of function may have artificially resulted in lower weight gains. Larger numbers of subjects are required to substantiate any statistical relevance.

The analysis of human plasma cytotoxicity to a porcine cell line (LLC-PK1) by MTT assay, both before and after immunoadsorption, showed a greater reduction with Synsorb 90 treatment than with controls (Appendix 4: Figure 7). Although this loss of cytotoxicity was not statistically significant, there is an apparent trend towards reduced cytotoxicity with Synsorb 90 immunoadsorption. It is possible that with a greater number of subjects in each group, a level of significance may be attained. An alternative hypothesis should also be considered: additional anti-porcine specific antibodies, targeted against other antigenic determinants, must exist.[84,91,162,164] This is an appealing theory, and the findings of low XNA titres following ELISA, without an equally significant change in cytotoxicity, appear to corroborate this (Appendix 4: Figure 6, 7). Further, the fact that XNA-depleted organs demonstrated prolonged survival and improved function in this study would suggest that the *in vivo* importance of alternative XNA and xenoantigenic sites remains unclear. These natural antibodies may or may not be critical in mediating the HAR response, just as their potential role in delayed xenograft rejection is, as yet, unknown.

Based on the results reported here, Synsorb 90 extracorporeal immunoadsorption may represent a feasible method for eliminating XNA in xenograft recipients. This could be combined with other therapies, such as complement

inhibition, and immunosuppressive drugs. While the immunobiology of xenograft transplantation continues to be investigated, the advances of scientific knowledge have not, as yet, obviated the need for pharmacological immunosuppressive therapy. Newer drugs that inhibit B-cell function, such as rapamycin, mycophenolate mofetil, and leflunomide,[226] reduce antibody production, and may be beneficial for xenograft survival. Such agents would act synergistically with Synsorb 90 in eliminating the effects of XNA. Recipients of transgenic organs may benefit from specific antibody depletion, as the long term effects of XNA presence are unclear. Additionally, XNA may impact negatively on myocardial function, through an electrophysiological mechanism.[248]

In summary, the results reported here demonstrate improved *ex vivo* cardiac xenograft survival with Synsorb 90 immunoadsorption to remove XNA. Significant prolongation of survival and improvement in function were attained without active inhibition of complement. Synsorb 90 immunoadsorption is highly efficacious in XNA removal, and may be an important adjunct to other necessary modalities in an effort to ameliorate hyperacute rejection.

APPENDIX 1: CLINICAL XENOTRANSPLANTATION

Renal Xenografts

Table 1: **Reported Clinical Attempts at Renal Xenotransplantation**

(Modified from Markmann & Barker[30])

Date	*Surgeon*	*Donor*	*(n)*	*Graft Survival*
1906	Jaboulay	Pig	1	3 days
1910	Unger	Goat	1	3 days
1923	Neuhof	Sheep	1	9 days
1964	Reemtsma	Chimpanzee	12	< 9 months
		Monkey	1	10 days
1964	Hitchcock	Baboon	1	5 days
1964	Starzl	Baboon	6	< 2 months
1964	Hume	Chimpanzee	1	1 day
1964	Traeger	Chimpanzee	3	2 months
1965	Goldsmith	Chimpanzee	2	4 months
1966	Cortesini	Chimpanzee	1	1 month

Cardiac Xenografts

Table 2: **Reported Clinical Attempts at Cardiac Xenotransplantation**
(Modified from Markmann & Barker[30])

Date	*Surgeon*	*Donor*	*(n)*	*Graft Survival*
1964	Hardy	Chimpanzee	1	0 days
1968	Cooley	Sheep	1	0 days
1968	Ross	Pig	1	0 days
1969	Marion	Chimpanzee	1	0 days
1977	Barnard	Chimpanzee	1	4 days
		Baboon	1	0 days
1984	Bailey	Baboon	1	20 days

Hepatic Xenografts

Table 3: **Reported Clinical Attempts at Hepatic Xenotransplantation**
(Modified from Markmann & Barker[30])

Date	*Surgeon*	*Donor*	*(n)*	*Graft Survival*
1966- 73	Starzl	Chimpanzee	3	< 14 days
1992-93	Starzl	Baboon	2	< 70 days
1992	Makowka	Pig	1	1 day

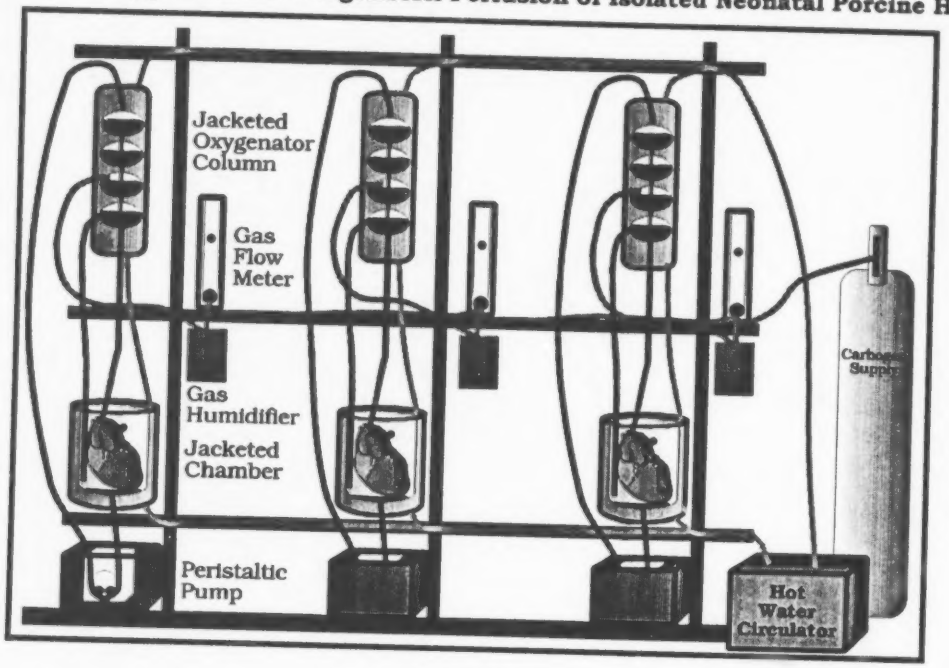
APPENDIX 2: IMMUNITY & ANTIBODY-COMPLEMENT

Table 4: **Antibody and Complement in Xenogeneic Donor-Recipient Combinations**
(From Lu et al.[40])

Recipient	Donor	Graft-type	*Natural Immunity* Complement Cascade (natural Antibody) Alternative	Classical	*Acquired Immunity* Antibody	Cellular
human	human (ABO compatible)	allograft	no	no	yes	yes
human	human (ABO incompatible)	allograft	no	yes	yes	yes
human	baboon chimpanzee	concordant xenograft	no	yes	yes	
human	pig	discordant. xenograft	no	yes	yes	yes
rat	guinea-pig	discord ant xenograft	yes	yes	yes	yes
human	rabbit	discordant xenograft	yes	yes	yes	yes

APPENDIX 3: EX VIVO PERFUSION MODEL

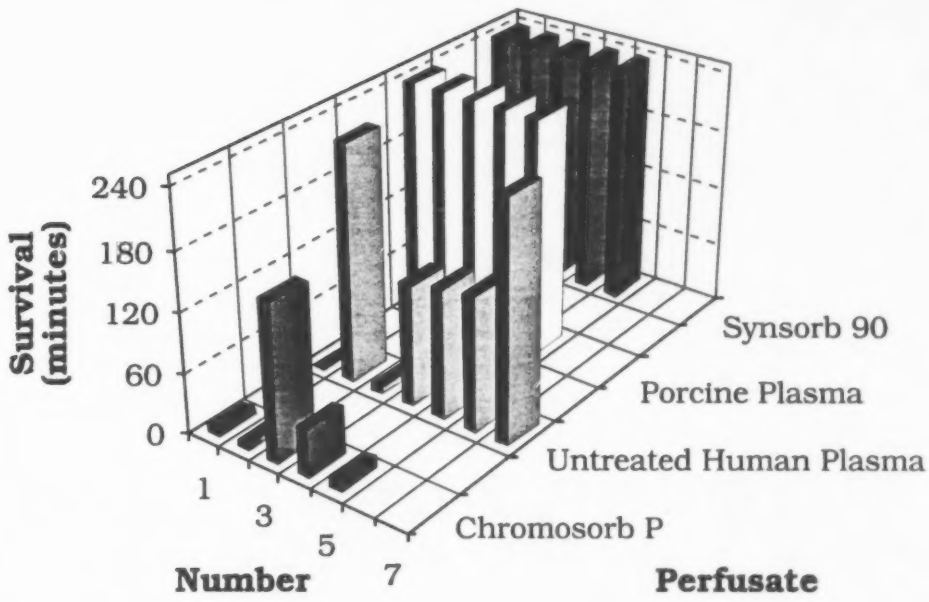
Figure 1: Apparatus for Langendorff Perfusion of Isolated Neonatal Porcine Hearts



Three independent experiments were performed simultaneously on a common manifold. After the heart was placed in the reservoir, the aorta was cannulated for Langendorff perfusion. A peristaltic pump recirculated blood products through a heat exchanger and oxygenator, which was placed at a constant height of 80 cm above the level of the coronary arteries.

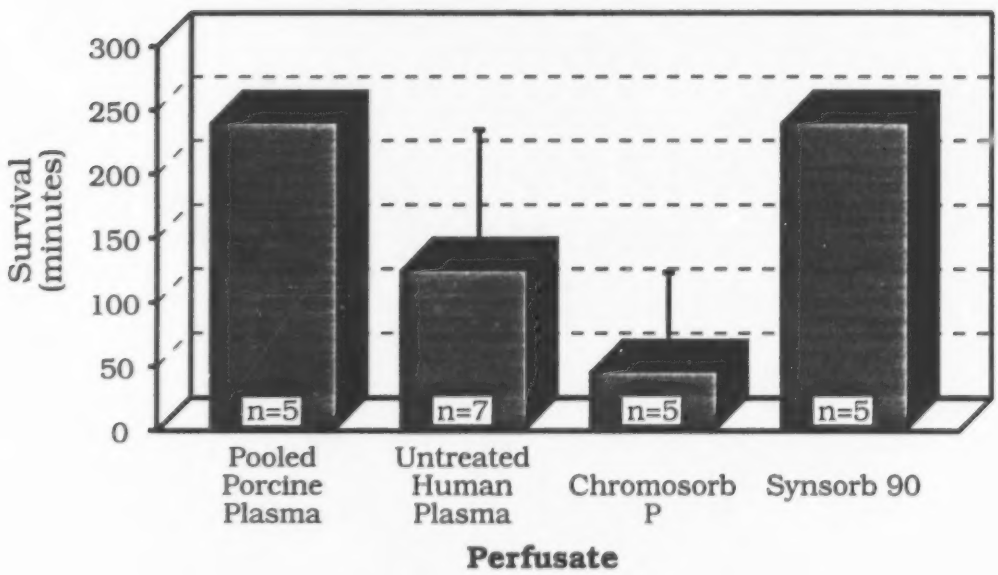
APPENDIX 4: DATA

Figure 2: Survival Times of Isolated Neonatal Perfused Heart Experiments



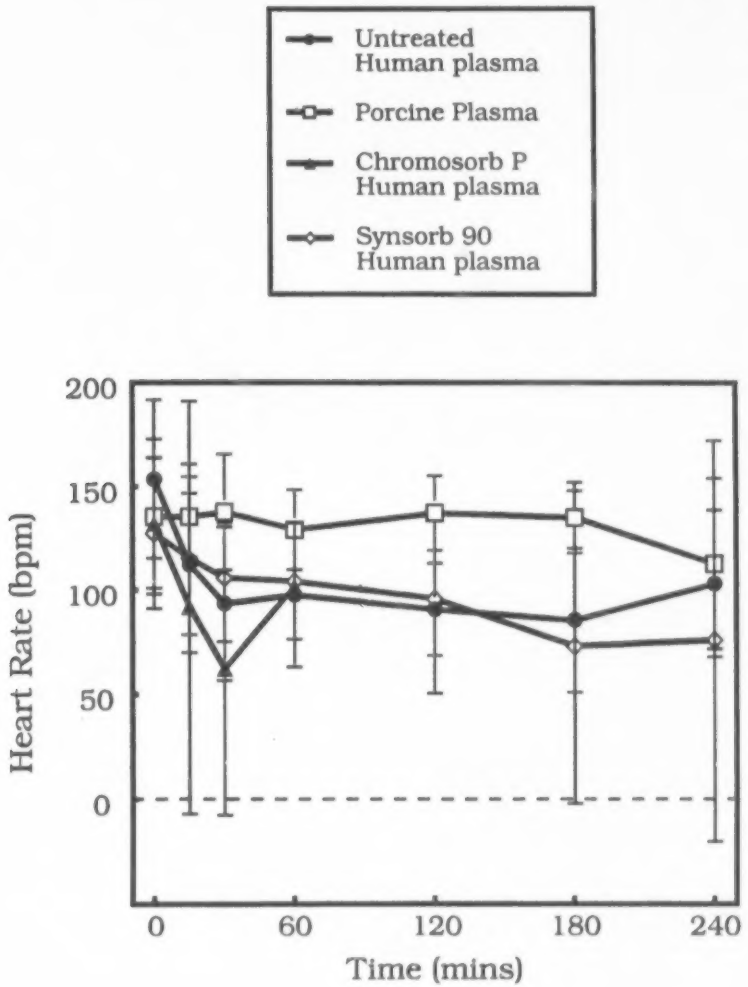
Survival times of individual isolated neonatal porcine perfused heart experiments, based on perfusate. Two of the hearts in the untreated group continued to beat for 240 minutes, but the relative function of this group remained poor. The Synsorb 90 arm demonstrated 240 minute survival in all cases. ANOVA of survival times confirmed the statistical significance of these data ($p<0.005$).

Figure 3: Mean Survival Times of Isolated Perfused Neonatal Porcine Hearts



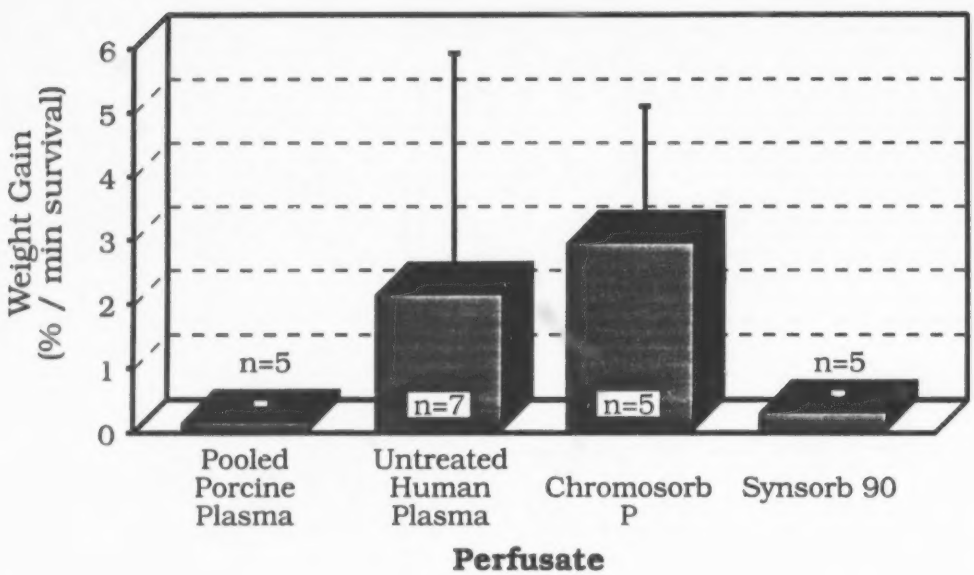
Survival times (mean ± standard deviation) of isolated perfused neonatal porcine hearts based on perfusate. *Ex vivo* perfusion was performed for 240 minutes, or until organ failure occurred. ANOVA revealed $p<0.005$, and post hoc comparisons confirmed that the Synsorb 90 and Chromosorb P groups differed significantly ($p<0.05$).

Figure 4: Mean Heart Rates During *Ex vivo* Perfusion Experiments



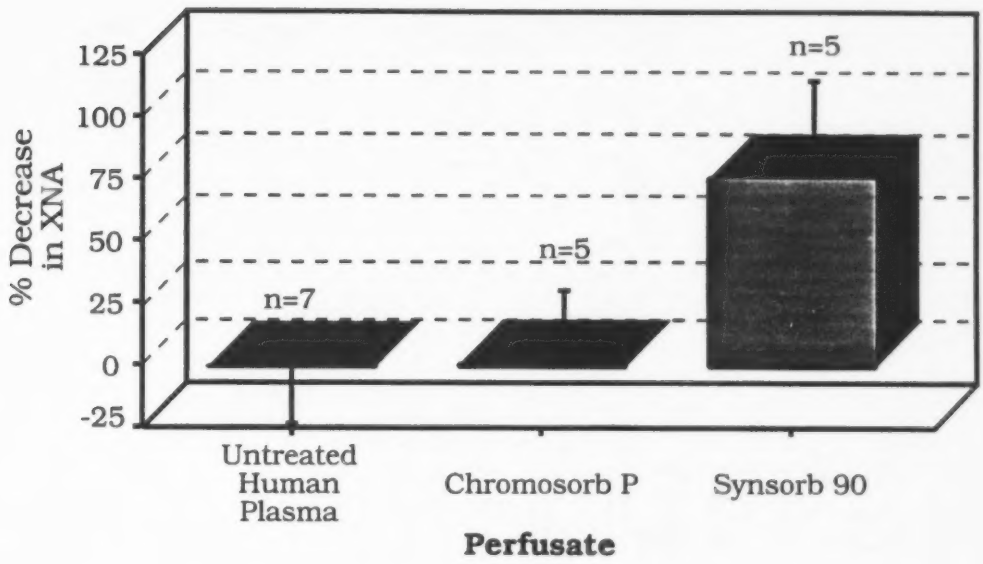
Heart rates (mean ± standard deviation) over the course of the *ex vivo* perfusion experiments by perfusate group. Porcine plasma hearts had a mean rate of 132 bpm (n=5), compared to Synsorb 90 with a mean rate of 105 bpm (n=5). Both Chromosorb P (n=5) and untreated human plasma (n=7) mean heart rates were 97 bpm. It is noteworthy that only 2 hearts in the untreated human plasma group survived beyond 135 minutes, thus skewing this curve.

Figure 5: Accumulation of Edema During *Ex vivo* Perfusion



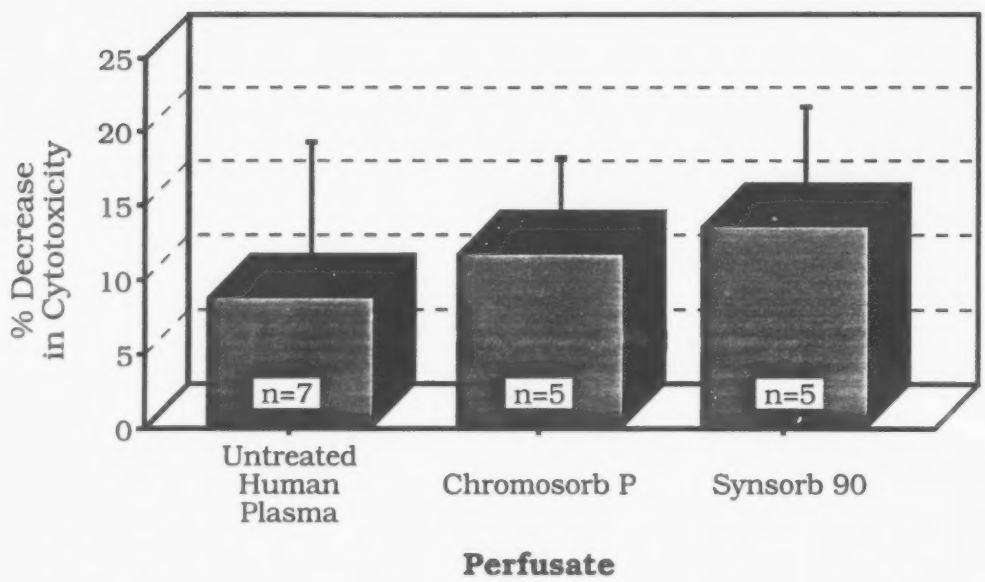
Accumulation of edema over the course of ex vivo perfusion as measured by weight gain per minute of organ survival ($p<0.005$ amongst groups). Untreated and Chromosorb P perfused hearts gained more weight than porcine plasma and Synsorb 90 perfused organs. This was significant at $p<0.01$ by post hoc comparisons. All results are expressed as mean ± standard deviation.

Figure 6: Mean Percent Change in XNA Following Immunoadsorption



Percent change (mean ± standard deviation) in XNA following immunoadsorption prior to isolated heart perfusion. Anti-pig IgG and IgM titres were assayed in 1:10 dilutions by direct ELISA against the α-Gal carbohydrate hapten bovine serum albumin (BSA) conjugate. The difference amongst groups was highly significant ($p<0.001$).

Figure 7: Mean Percent Change of Cytotoxicity Following Immunoadsorption



Percent change in human serum cytotoxicity (mean ± standard deviation) following immunoadsorption with Chromosorb P and Synsorb 90, compared to untreated human plasma control. Plasma samples prior to immunoadsorption and following treatment, immediately preceding *ex vivo* perfusion, were examined in 1:4 dilutions by MTT cytotoxicity assays.

Table 5: C3 and C4 Levels

LEGEND:

UnRx = untreated human plasma
C/P = human plasma adsorbed with Chromosorb P
S-90 = human plasma adsorbed with Synsorb 90

All values reported as g/L

Group	C3 Pre-Adsorption	C3 Pre-Perfusion	C3 Post-Perfusion	C4 Pre-Adsorption	C4 Pre-Perfusion	C4 Post-Perfusion
UnRx	1.037±0.143	1.003±0.146	0.898±0.200	0.255±0.097	0.265±0.077	0.255±0.083
C/P	0.791±0.142	0.686±0.109	0.628±0.089	0.171±0.008	0.129±0.011	0.120±0.012
S-90	0.898±0.238	0.659±0.151	0.572±0.052	0.224±0.047	0.168±0.036	0.138±0.025

C3 Reference Range = (0.75-1.9) g/L

C4 Reference Range = (0.18-0.36) g/L

C3 and C4 levels (measured in g/L) were detectable in significant quantities, even following immunoadsorption and ex vivo perfusion. All results are expressed as mean values ± standard deviation. The normal laboratory reference ranges are given below the main table.

Table 6: Semi-Quantitative Immunohistochemical Analysis

LEGEND:

UnRx = untreated human plasma
C/P = human plasma adsorbed with Chromosorb P
S-90 = human plasma adsorbed with Synsorb 90
PP = porcine plasma

Group	*IgA*	*IgM*	*IgG*
UnRx	1.85±0.69	2.85±0.69	0.85±0.69
C/P	2.14±0.69	3.28±0.49	0.85±0.69
S-90	1.00±0.82	2.28±0.75	0.57±0.53
PP	0±0	0±0	0±0

Endothelial staining detected on immunohistochemical analysis was scored on a scale of 0 - 4+ by two independent examiners, in a blinded fashion. All values are expressed mean ± standard deviation. IgA and IgM binding in Synsorb 90 perfused hearts differed from the Chromosorb P group ($p<0.02$) by Kruskal-Wallis rank sum ANOVA and post hoc testing. A similar trend was noted with IgG.

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